

# The Herpetological Journal

Volume 31, Number 2

April 2021



Published by the British Herpetological Society

# British Herpetological Society



*The Herpetological Journal is published quarterly by the British Herpetological Society and is issued free to members. Articles are listed in Current Awareness in Biological Sciences, Current Contents, Science Citation Index and Zoological Record. Applications to purchase copies and/or for details of membership should be made to the Hon. Secretary, British Herpetological Society, The Zoological Society of London, Regent's Park, London, NW1 4RY, UK. Instructions to authors are printed inside the back cover. All contributions should be addressed to the Scientific Editor.*

*Front cover: Dusky rattlesnake (Crotalus triseriatus) in Mexico.  
See the article on page 91.*

## Copyright

It is a fundamental condition that submitted manuscripts have not been published and will not be simultaneously submitted or published elsewhere. By submitting a manuscript, the authors agree that the copyright for their article is transferred to the publisher if and when the article is accepted for publication. The copyright covers the exclusive rights to reproduce and distribute the article, including reprints and photographic reproductions. Permission for any such activities must be sought in advance from the Editors.



Contents

Full papers	
Genetic differentiation over a small spatial scale in the smooth newt ( <i>Lissotriton vulgaris</i> ) <i>Zoltán Tóth, Fanni Mihók &amp; Edina Nemesházi</i>	61-69
Thermoregulation comparisons between a threatened native and an invasive lizard species <i>Yole Caruso, Daniele Macale, Luca Luiselli &amp; Leonardo Vignoli</i>	70-76
Occurrence of lizards in agricultural land and implications for conservation <i>Marta Biaggini &amp; Claudia Corti</i>	77-84
Influence of the presence of invasive mosquitofish and submerged vegetation on oviposition site selection by gray treefrogs ( <i>Hyla versicolor</i> ) <i>Geoffrey R. Smith &amp; Jessica E. Rettig</i>	85-90
Hidden in plain sight: detectability and habitat selection of the central plateau dusky rattlesnake in anthropized landscapes <i>Leroy Soria-Díaz, Claudia C. Astudillo-Sánchez, Yuriana Gómez-Ortiz, Javier Manjarrez, Víctor Mundo-Hernández, Tamara Rubio-Blanco &amp; Hublester Domínguez-Vega</i>	91-98
Can ventral pattern be used for individual recognition of the vulnerable Pyrenean brook newt ( <i>Calotriton asper</i> )? <i>Manon Dalibard, Laëtitia Buisson, Olivier Calvez, Morgan Nguyen-Hong, Audrey Trochet &amp; Pascal Laffaille</i>	99-110
How reliable is the habitat suitability index as a predictor of great crested newt presence or absence? <i>Andrew S. Buxton, Hannah Tracey &amp; Nick C. Downs</i>	111-117



Published by the British Herpetological Society

Genetic differentiation over a small spatial scale in the smooth newt (*Lissotriton vulgaris*)

Zoltán Tóth<sup>1</sup>, Fanni Mihók<sup>2,3</sup> & Edina Nemesházi<sup>4,5,6</sup>

<sup>1</sup>Department of Zoology, Plant Protection Institute, Centre for Agricultural Research, ELKH, Budapest, Hungary  
<sup>2</sup>University of Veterinary Medicine, Budapest, Hungary  
<sup>3</sup>Faculty of Electrical Engineering and Informatics, Budapest University of Technology and Economics, Budapest, Hungary  
<sup>4</sup>Konrad Lorenz Institute of Ethology, Department of Interdisciplinary Life Sciences, University of Veterinary Medicine, Vienna, Austria  
<sup>5</sup>Lendület Evolutionary Ecology Research Group, Plant Protection Institute, Centre for Agricultural Research, ELKH, Budapest, Hungary  
<sup>6</sup>Conservation Genetics Research Group, Department of Ecology, Institute for Biology, University of Veterinary Medicine, Budapest, Hungary

Understanding the distribution of genetic variation is central for both population biology and conservation genetics. Genetic population structure can be primarily affected by the species’ dispersal ability, which is assumed to be limited in many amphibians. In this study, we estimated allelic differentiation metrics and  $F_{ST}$  indices to investigate genetic variation among natural breeding ponds of smooth newts (*Lissotriton vulgaris*) over a small spatial scale. Based on six microsatellite loci, we found a small, but significant allelic differentiation among clusters of natural breeding ponds (i.e. ‘local regions’), which result was in line with the calculation of corresponding hierarchical  $F_{ST}$  values. Analysis of molecular variance also indicated significant between-region variation in the study area. Pairwise estimations showed that only the furthestmost regions differed from each other in both differentiation measures, but this difference was not attributable to geographic distances between ponds. Our results provide evidence that hierarchical genetic structure can be characteristic to breeding ponds of smooth newts on a small spatial scale in their natural breeding habitat, but dispersal distance may be less limited than previously thought in these philopatric caudates.

**Keywords:** genetic differentiation, natural habitats, isolation by distance, philopatric species, hierarchical diversity partitioning, fixation indices, Shannon entropy, *Lissotriton vulgaris*

INTRODUCTION

Genetic variation forms the basis for various micro-evolutionary processes and has a fundamental role in the long-term persistence of populations and species (Hoffmann & Willi, 2008). Because of that, investigating the distribution of genetic variation and prevalent genetic structure is important from both theoretical and applied perspectives (Holderegger et al., 2006; Petit et al., 1998; Smouse & Peakall, 1999). Studying genetic diversity in natural populations, for instance, may reveal those spatial characteristics that facilitate gene flow and influence the structural properties of migration/dispersal networks, and help to identify areas or populations that should be prioritised when allocating conservation efforts (Emel et al., 2019; Lesbarreres et al., 2006). Amphibians are among the most threatened vertebrate taxa worldwide (Arntzen et al., 2017; Powers & Jetz, 2019), so more and more investigations are being carried out on amphibian species to scrutinise how landscape characteristics affect genetic differentiation between breeding populations

at various spatial scales (Atlas & Fu, 2019; Almeida-Gomes & Rocha, 2014; Luqman et al., 2018). Other studies utilise genetic data to test whether amphibian populations actually exhibit metapopulation dynamics as a priori expected in many species; this knowledge is crucial for the appropriate conservation management of breeding locations (Marsh & Trenham, 2001; Watts et al., 2015; Billerman et al., 2019).

The smooth newt is one of the most widespread newt species in Europe (Arntzen et al., 2009), and a popular subject for the study of developmental plasticity (e.g. Martin et al., 2016; Tóth & Hettyey, 2018), mate choice (e.g. Secondi & Théry, 2014; Secondi et al., 2015) and hybridisation between sister species (e.g. Zieliński et al., 2019; Niedzicka et al., 2020). Based on findings of conventional mark-recapture studies, smooth newts are regarded to be highly philopatric, with females usually returning to their natal pond to reproduce (Bell, 1977), and adults and juveniles having very short dispersal distances (ranging between 50-182 metres; Griffiths, 1984; Warwick, 1949; Bell, 1977; Dolmen, 1981).

However, such poor dispersing ability may be unrealistic for many landscapes and seemingly contradicts the broad distribution of the species. Dispersal characteristics have important consequences on the spatial distribution of genetic variation. If dispersal is as limited as implied by the above empirical findings, isolation by distance (IBD) can be expected to shape genetic variation even on small spatial scales. IBD may lead to higher genetic similarity between pairs of populations that are close to each other compared to populations that are farther away from each other, without any selective advantage of such patterns (Meirmans, 2012; Diniz-Filho et al., 2013). On the other hand, limited dispersal may also increase differentiation in mean phenotype among populations, facilitating local adaptation (Blanquart et al., 2012; Arendt, 2015), or increase the risk of genetic drift that could, in some instances, counteract adaptation (Frankham et al., 2010). A more recent study based on the analysis of both capture-mark-recapture and genetic data did not support the observation of such limited dispersal in this species, however. In an agricultural area of small spatial scale (with 270–1800 metres between-pond distances), Schmidt et al. (2006) found overall low genetic differentiation in allozyme loci across five breeding population of smooth newts. They also showed that significant genetic differentiation was present only between some ponds that were at least 930 metres apart, but adult migration and gene flow occurred between other ponds that were even farther away. Similarly, low differentiation patterns can be expected in natural wooded landscapes, especially as shelters for all terrestrial stages, humidity at the ground level and the presence of temporary, small surface waters in natural habitats may increase survival probability and facilitate juvenile dispersal and/or between-pond migration of breeding adults. Furthermore, juveniles might disperse to much greater distances from the breeding ponds compared to adults (with a maximum of ca. 1000 metres; Müllner, 2001; Sinsch & Kirst, 2015).

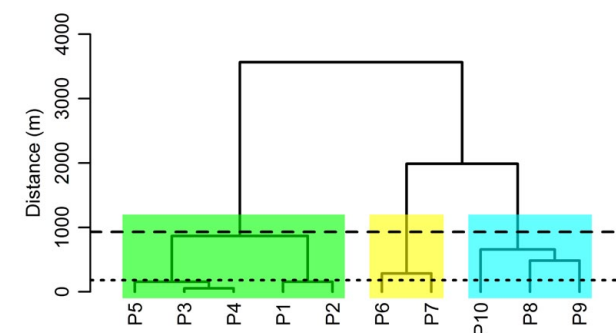
In this study, we characterised the distribution of genetic variation between breeding ponds of smooth newts (*Lissotriton vulgaris*) at a small spatial scale. We sampled adults from 10 water bodies located in an approx. 10 km<sup>2</sup> area of natural landscape during their reproductive period, and measured genetic diversity and estimated neutral genetic differentiation on the pond and local region (i.e. groups of ponds) levels using six microsatellite loci. For comparison, we also calculated  $F_{ST}$  values on the same hierarchical levels (Yang, 1998; Goudet, 2005; 2007). In accordance with the findings of Schmidt et al. (2006), we predicted that genetic differentiation would be observed, if at all, only at the local region level and isolation by distance would be negligible in the distribution of genetic variation within local regions on the studied spatial scale.

## MATERIALS AND METHODS

### Study area

The study area is located in the north-eastern part of

the Pilis-Visegrád Mountains, Hungary, and belongs to the operational area of the Danube-Ipoly National Park (Fig. 1, Table S1). Smooth newts regularly breed in 10 permanent and semi-permanent ponds located on an approx. 10 km<sup>2</sup> area of deciduous forests and natural clearings (Tóth et al., 2011; Tóth, 2015; Bókonyi et al., 2016). The area is also characterised by the presence of two secondary asphalt roads, as well as a few forestry dirt roads, and several temporary and semi-permanent watercourses. However, there were no other potential breeding ponds within the study area in the studied years to the best of our knowledge. The altitude of the sampled ponds varies between 254 and 538 metres above sea level. Ponds were grouped into three ‘local regions’ (‘Upper’ (U) region: P1, P2, P3, P4, P5; ‘Middle’ (M) region: P6, P7; ‘Lower’ (L) region: P8, P9, P10) based on the spatial distance between them and the estimated maximal dispersal distance of juvenile smooth newts (Fig. 1). Within-region distance (measured as path lengths taking the variation in altitude between ponds also into account) ranged between 55.1 and 866.9 metres, while the distance among ponds belonging to different regions varied between 1320.4 and 3566.4 metres (Table S1; Fig. S1).



**Figure 1.** Dendrogram from the hierarchical clustering of ponds based on between-pond distances. Dashed line indicates the threshold value of 930 metres, which corresponds to the minimum distance between breeding sites of smooth newts that were found to be genetically differentiated by Schmidt et al. (2006). We used this value to define local regions in our study: ponds belonging to the ‘Upper’ local region are shown with a green background, ponds belonging to the ‘Middle’ local region are marked with a yellow background, and ponds belonging to the ‘Lower’ local region are illustrated with a cyan background, respectively. Dotted line shows the largest reported dispersal distance of 182 metres reviewed in Smith & Green (2005).

### Sample collection and DNA extraction

We captured smooth newts during their breeding season (March–April) using underwater traps and by dip-netting in 2014 and 2015. In 2014, gravid females were collected from ponds P3, P6, P8 and P9 (Table S1; Tóth, 2015), while in 2015, both females and males were caught from all 10 ponds. We brought the animals to

the laboratory in individual plastic boxes appropriate for transportation. We anaesthetised the individuals by inserting them into a 0.2 % solution of MS-222 (CAS: 886-86-2, Sigma-Aldrich Co., USA), then we collected swab samples from them using buccal swabs (Goldberg, Kaplan & Schwalbe, 2003; Pidancier, Miquel & Miaud, 2003). Animals were anaesthetised in order to reduce the risk of injury during swab sampling and to take photographs of the collected individuals. Samples were stored at 4 °C until DNA extraction. DNA was extracted and purified using the QIAamp DNA Investigator Kit (QIAGEN N.V., Venlo, The Netherlands), following the protocol of the manufacturer. The concentration and purity of extracted DNA was estimated using NanoDrop. We evaluated fifteen microsatellite loci that were previously described and used for population genetics analyses in this species: Tv3Ca9, Tv4Ca9, Tv5Ca13 (Johanet et al., 2009), Lm\_749, Lm\_528, Lm\_632, Lm\_521, Lm\_013, Lm\_870, Lm\_488 (Nadachowska, Flis and Babik, 2010), Lm\_346, Lm\_AHNC3 (Nadachowska-Brzyska et al., 2012), Lm\_ZN5, Lm\_TDP, Lm\_8BH (Zieliński et al., 2013). Primer synthesis, multiplex labeling PCR and fragment analyses were performed by Biomi Ltd. (Gödöllő, Hungary) following the protocol reported in Zieliński et al. (2013). The PCR products were electrophoresed on a 3130xl Genetic Analyzer with GeneScan 500 LIZ size standard, and GeneMarker v2.7.0 (fully functional validation version; SoftGenetics, State College, PA, USA) was used for manual allele scoring. Six out of the 15 evaluated loci (Lm\_013, Lm\_528, Lm\_870, Lm\_488, Lm\_ZN5, Lm\_TDP) yielded very low or ambiguous peaks (likely due to the low quality/quantity of DNA obtained from the swabs samples or suboptimal ratio of microsatellite PCR products in the product mix, relative to each other), and therefore were discarded from the study.

### Genetic and statistical analysis

We used the tandem 1.09 software (Matschiner & Salzburger, 2009) for the automatic binning of microsatellite allele sizes in the nine loci that produced reliable amplifications. As we found higher average rounding error in terms of allele size (i.e. many detected fragment lengths could not be classified unambiguously as one or another allele defined in base pairs) compared to the recommended threshold value in additional three loci (Lm\_521, Lm\_632 and Tv3Ca9), all further analyses were conducted using only the remaining six loci (Table S2). This way, we could ensure that ambiguously identified allele sizes would not bias our estimations of genetic differentiation between ponds. We checked for stuttering and null alleles using Micro-Checker 2.2.3 (Van Oosterhout et al., 2004). Probability of identity ( $P_{ID}$ ) of increasing numbers of loci and pairwise estimators of relatedness (Lynch & Ritland, 1999) between individuals were calculated with Genalex 6.503 (Peakall & Smouse, 2012). The number of alleles (A), allelic richness (Ar; calculated for each locus as the number of alleles divided by the number of samples without missing data at that locus), observed and expected heterozygosity ( $H_o$  and  $H_e$ , respectively) were computed using the ‘strataG’ R

package (Archer et al., 2016). Tests of Hardy-Weinberg equilibrium (HWE) and tests for linkage disequilibrium (LD) were calculated in each sampled breeding pond using Genepop 4.7.0 (Rousset, 2008); we applied the Bonferroni procedure to control for type I error.

Genetic diversity in the hierarchically structured ecosystem-region-pond system (i.e. all sampled ponds, groups of ponds and individual ponds, respectively) was decomposed based on the calculation of Hill numbers of order 1, which weights all elements in proportion to their frequency and leads to diversity measures based on Shannon’s entropy (Jost, 2006; 2008; Jost et al., 2018; Gaggiotti et al., 2018). Gamma (on the ecosystem level), alpha and beta (both on the local region and pond levels) diversity components were calculated separately for each locus using the ‘iDIP’ function in the supplementary R script published by Gaggiotti et al. (2018). As beta diversities depend on both the actual number of local regions/ponds and their weights (number of individuals sampled within each region/pond), we also calculated normalised differentiation indices ( $\Delta D$ ) to quantify compositional differentiation at given hierarchical levels. We used the average values of these measures over the six loci to characterise region- and pond-level allelic differentiation as in Gaggiotti et al. (2018). We also calculated hierarchical  $F_{ST}$  values and  $F_{ST}$  analogues as ‘fixation’ measures; such metrics, being sensitive only to demographic variables, reflect the degree of completion toward fixation and not the actual degree of differentiation of allele frequencies between populations (Jost et al., 2018). We computed hierarchical  $F_{ST}$  values on the local region and pond levels for comparison using the ‘hierfstat’ R package (Goudet & Jombart, 2015). We used permutation tests with 9999 iterations to examine if the observed differentiation measures were different from the ones estimated from permutation distributions, which were created by reshuffling individuals among ponds and local regions. In the case of  $F_{ST}$ , the best statistics to test for differentiation is proposed to be the likelihood ratio G-statistics (Goudet et al., 1996; Goudet, 2005; De Meeûs & Goudet, 2007). We used the in-built ‘test.g’ function of the ‘hierfstat’ package (which is equivalent to the ‘test.between’ function with the level of randomisation set to the level of individuals) to test the significance of given hierarchical levels on genetic differentiation. In order to uncover which local regions differed from each other, we estimated region-level differentiation measures between pairs of local regions, and compared the observed values to corresponding permutation distributions. One-tailed conservative P-values were calculated as  $(b+1)/(m+1)$ , where  $b$  is the number of permutation test statistics (either  $G^*$  [i.e. multilocus G-statistics] or  $\Delta D$  values) equal or greater than the observed ones, and  $m$  is the number of iterations (Phipson & Smyth, 2010). Analysis of molecular variance (AMOVA) was also used to compute the ratio of variance components obtained from a matrix of squared Euclidean distances between pairs of individuals; this analysis represents an alternative computational method to test possible differences in a nearness to fixation measure ( $\Phi$



statistics) among different hierarchical levels (Excoffier et al., 1992; Meirmans, 2006). AMOVA components were tested for significance with 9999 permutations. We calculated Nei's pairwise  $F_{ST}$  values between ponds, and tested for IBD by investigating the correlation between linearised pairwise  $F_{ST}$  measures (Rousset, 1997) and the logarithm of geographical distances using Mantel's test (Jensen et al., 2005). For these latter tests, we used the relevant functions of the 'poppr' (Zamvar et al., 2014) and 'vegan' (Oksanen et al., 2019) R packages, respectively. We also estimated the number of genetically different clusters of ponds by conducting a Bayesian cluster analysis in GENELAND (Guillot et al., 2005, 2012). We used spatial explicit Bayesian model based on simulations of microsatellite data and geographic information on pond locations; MCMC simulations consisted of 2000000 iterations with a thinning of 1000 correlated allele frequencies and a burn-in of 200. We performed 10 independent runs and based our inference on the run giving the highest average posterior probability as suggested in the GENELAND manual. Descriptive statistics and differentiation measures were calculated and statistical tests (except the tests of HWE and for LD) were performed in R 3.6.1 (R Core Team, 2019).

RESULTS

We found no evidence of scoring error due to stuttering, large allele dropout or null alleles in the six loci. Probability of identity was estimated at  $1.765 \times 10^{-7}$ , being threefold below the conservative threshold of  $P_{ID} \leq 0.0001$  (Waits et al., 2001), whereas the average pairwise relatedness among the sampled individuals was  $-0.005 \pm 0.071$  (mean  $\pm$  SD) with a maximum value of 0.415. Thus, we concluded that we sampled the individuals only once and our dataset was adequate for estimating genetic diversity in the study area. The number of alleles per locus ranged from 5 (Tv4Ca9) to 27 (Lm\_749) with expected heterozygosity values in the range of 0.622–0.936 (Table S2). After applying Bonferroni correction for multiple tests, we detected no significant LD for any locus pair or deviation from HWE for any loci in any of the sampled ponds.

The effective numbers of alleles calculated for each locus are shown in Table 1. Allelic differentiation across loci was found to be 0.065 on the local region-level, and 0.114 among ponds within a region. Despite the higher absolute value of this differentiation index on the pond level, permutation tests revealed that allelic differentiation was higher than expected by chance among local regions ( $P=0.015$ ), but not among ponds within a region ( $P=0.907$ ). The region-level  $F_{ST}$  was calculated to be 0.010, while the pond-level  $F_{ST}$  was 0.003; corresponding tests also indicated that differentiation was significantly higher than expected by chance only at the regional level (region-level  $G^*=170.65$ ,  $P=0.019$ ; pond-level  $G^*=536.01$ ,  $P=0.326$ ). In pairwise comparisons, we found that only the 'Upper' (U) and 'Lower' (L) local regions differed significantly in terms of allelic differentiation, whereas there was only a weak tendency

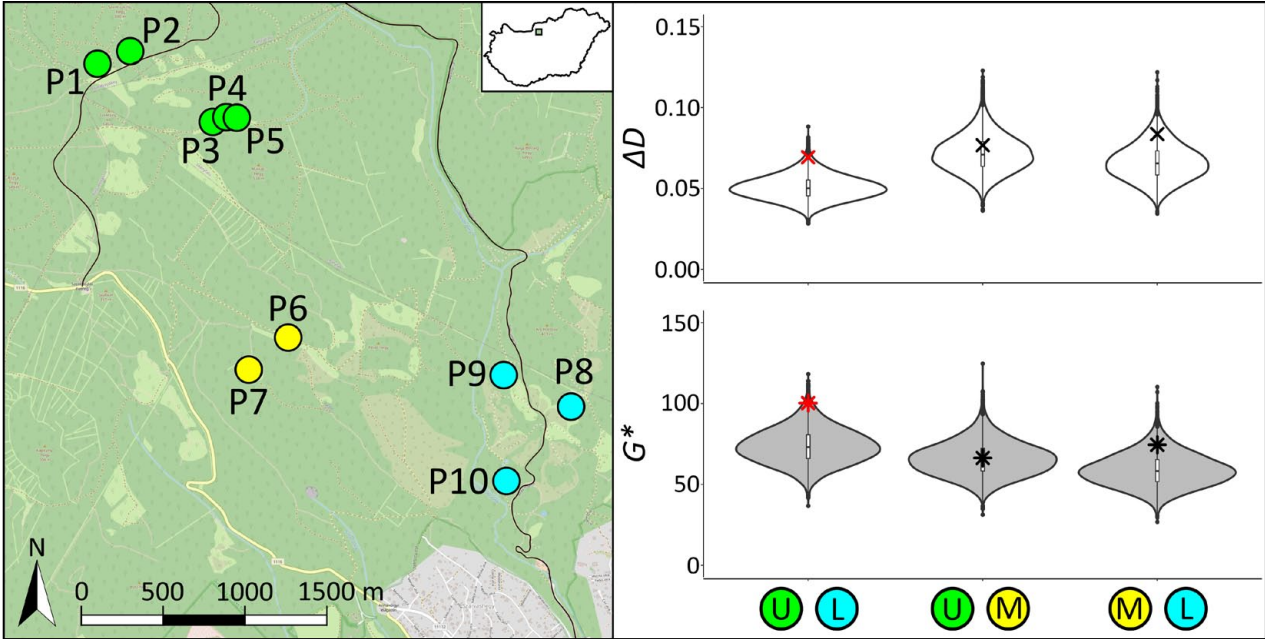
**Table 1.** Decomposition of genetic diversity in the study area.  $D_{\gamma}^{total}$  indicates gamma diversity on the ecosystem level, while  $D_{\alpha}$  denotes alpha diversity and  $D_{\beta}$  beta diversity, respectively. Upper-case numbers indicate the hierarchical level at which given components were estimated (1: pond-level, 2: local region-level).  $\Delta D$  denotes normalised differentiation indices. Values within the parentheses are one-tailed conservative  $P$ -values; significant differences are shown in bold.

Loci	$D_{\gamma}^{total}$	$D_{\alpha}^{(2)}$	$D_{\alpha}^{(1)}$	$D_{\beta}^{(2)}$	$D_{\beta}^{(1)}$	$\Delta D^{(2)}$	$\Delta D^{(1)}$
L1	18.414	15.987	11.175	1.152	1.431	0.136 (0.465)	0.289 (0.852)
L2	3.936	3.806	3.602	1.034	1.057	0.032 (0.099)	0.045 (0.778)
L3	4.793	4.524	4.101	1.060	1.103	0.056 (0.045)	0.079 (0.549)
L4	3.266	3.167	2.892	1.031	1.095	0.030 (0.638)	0.074 (0.289)
L5	5.139	4.717	4.160	1.090	1.134	0.083 (0.015)	0.101 (0.785)
L6	5.258	4.974	4.426	1.057	1.124	0.053 (0.159)	0.095 (0.665)

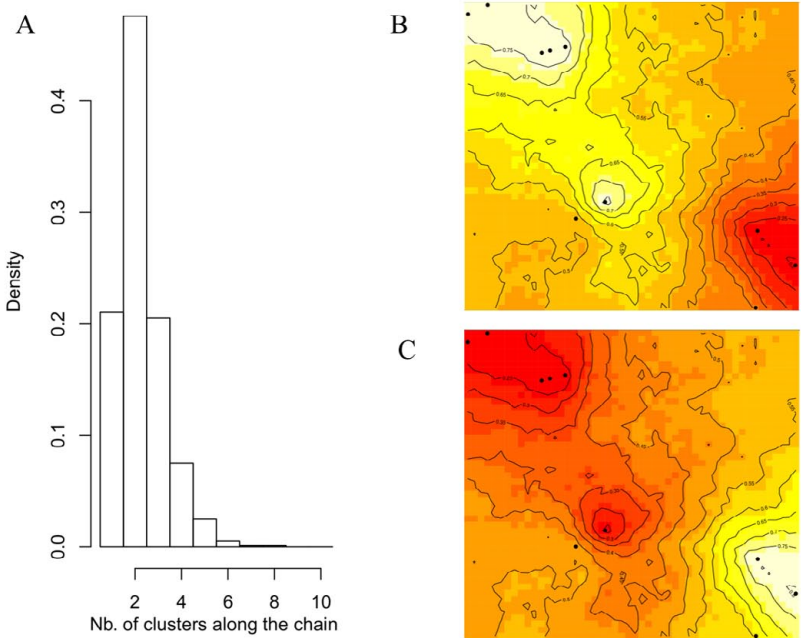
in difference between the 'Middle' (M) and 'Lower' local regions in this measure (U-L:  $\Delta D=0.069$ ,  $P=0.011$ ; U-M:  $\Delta D=0.077$ ,  $P=0.307$ , M-L:  $\Delta D=0.084$ ,  $P=0.073$ ; Fig. 2). Similarly, region-level  $G^*$  indicated that the 'Upper' and 'Lower' local regions were significantly different in terms of nearness to fixation as well, while difference between the 'Middle' and 'Lower' regions was marginally non-significant (U-L:  $G^*=100.24$ ,  $P=0.009$ ; U-M:  $G^*=66.33$ ,  $P=0.463$ ; M-L:  $G^*=74.48$ ,  $P=0.070$ ). In accordance with the above tests, AMOVA showed that local regions were genetically differentiated from each other ( $\Phi=0.015$ , Sum of Squares=6.717,  $d.f.=2$ ,  $P=0.018$ ), with 1.5 % of genetic variation distributed among regions, while variation among ponds within regions was essentially zero ( $\Phi=-0.010$ ,  $-0.96\%$ ; Sum of Squares=15.323,  $d.f.=7$ ,  $P=0.824$ ). MCMC simulations consistently retrieved two genetically different populations at two clusters of ponds (Fig. 3), indicating the presence of genetic differentiation between the 'Upper' and 'Lower' local regions that was also found in the permutation tests. We found no correlation between genetic and geographic distances between ponds (Mantel's test,  $r=-0.001$ ,  $P=0.484$ ; Fig. 4).

DISCUSSION

We examined the extent of genetic differentiation on the pond and local region levels between demes of smooth newts over a scale of about 3.5 km in a natural breeding habitat. We found that genetic differentiation on the local region level was significantly higher than what we would expect by chance, and this result was supported by the calculation of two differentiation measures and by the analysis of microsatellite variance. Pairwise comparisons of local regions suggested that this region-level differentiation was due to difference in both the actual allelic composition and estimated nearness to fixation (measured by hierarchical  $F_{ST}$  and  $\Phi$ ) between the



**Figure 2.** Locations of the breeding ponds (left panel) and null distributions of regional  $\Delta D$  (normalised allelic differentiation index) and  $G^*$  (multilocus G-statistics) with the observed values for each pair of local regions (right panel). Ponds marked with different colours belong to different local regions (U: 'Upper' region, M: 'Middle' region, L: 'Lower' region). Violin plots indicate the distribution of differentiation indices (white:  $\Delta D$ , grey:  $G^*$ ) calculated from reshuffled datasets reduced to the two regions in question. Symbols (x:  $\Delta D$ , \*:  $G^*$ ) denote the observed values calculated from the original data. Symbols in red indicate significant deviations from the corresponding permutation distributions. Between-region pond distances are shown in Table S1 and Figure S1. The map was created using OpenStreetMap (OpenStreetMap contributors 2015).

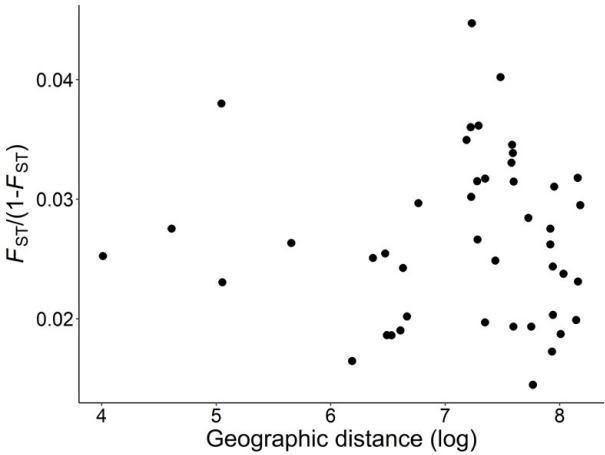


**Figure 3.** Bayesian clustering analysis conducted in GENELAND. **A)** Distribution of posterior probability of a number of genetic clusters (K). **B, C)** Maps of population membership probabilities for 107 smooth newts belonging to one of the two inferred clusters.

two furthestmost regions. Genetic distance, however, did not correlate with geographic distance between ponds, indicating that isolation by distance was not likely to be the major cause of the detected genetic differentiation among local regions. Our results support the previous findings of Schmidt

et al. (2006) regarding low genetic variation in smooth newts on the studied spatial scale, and confirm that dispersal distance between demes may be far greater than implied by earlier studies (50-182 metres; e.g. Griffiths, 1984; Bell, 1977; Dolmen, 1981). As we found no significant genetic differentiation between the





**Figure 4.** Relationship between genetic differentiation expressed as transformed  $F_{ST}$  and logarithm of geographic distances for the ten breeding ponds of smooth newts in the study area.

‘Upper’ and ‘Middle’ regions (this was also confirmed by the performed Bayesian clustering analysis), individuals were likely to be able to move among suitable breeding sites that are ca. 1.4 – 2 km apart under suitable weather conditions (e.g. on rainy days, as suggested by Weddelling et al., 2004). On the other hand, distances of 2.3–3.6 km between ponds in the ‘Upper’ and ‘Lower’ regions generated detectable differences between demes in the study area. Still, the estimated value of region-wise  $F_{ST}$  (0.010) was only a fraction of what is usually considered biologically significant because of reduced gene flow between populations (Frankham et al., 2010). Allelic differentiation measures also indicated that the number of local region equivalents was close to unity for most loci, and only 1.5 % genetic variation was distributed among regions. Our findings thus support and provide further genetic evidence for the idea that dispersal distance is likely to be underestimated in smooth newts and, in fact, individuals may regularly disperse between ponds, with some animals covering great distances and connecting apparently separate breeding populations (as suggested for many amphibians; Marsh & Trenham, 2001; Smith & Green, 2005). Although differentiation in fixation measures was found to be higher in some *Lissotriton* species than the estimated value of regional  $F_{ST}$  in this study, these discrepancies were rather related to the spatial scales on which those studies were conducted. For instance, in *L. graecus*, Sotiropoulos et al. (2013) showed that the overall genetic differentiation among demes at 10 breeding ponds in a semi-natural landscape was moderate ( $F_{ST}$  with 95 % CI: 0.039 [0.011, 0.092]), with geographical distance between localities ranging between c.a. 0.05 and 6.3 km. In the *L. vulgaris meridionalis*, Buono et al. (2017) showed that pairwise  $F_{ST}$  values between three locations that were 10.1–15.7 km far from each other ranged between 0.081 and 0.132. Previous works have pointed out that allelic and fixation differentiation measures do not provide the same information about the genetic variation in populations, but quantify complementary aspects of the prevailing

genetic structure (Bird et al., 2011; Caballero & García-Dorado, 2013; Jost et al., 2018). Here we used both hierarchical  $F_{ST}$  and Shannon entropy-based  $\Delta D$  indices for estimating genetic differentiation. Because the results for the two types of measures were in agreement, we are confident that the detected small, but significant local region-level genetic differentiation reflects a genuine pattern of genetic variation between smooth newt breeding ponds across the study area. Our results highlight that limited dispersal distance in amphibians should not be assumed automatically, but needs to be measured directly/inferred to indirectly from genetic data in the species of interest. The capacity of a species to exchange individuals between (sub-)populations is often determined by an interaction between spatial distance and individual land use. Some habitat features can facilitate animals’ movement through a landscape. For example, water bodies that are unsuitable for reproduction still may serve as stepping stones connecting distant breeding sites, while habitat features representing barriers cause genetic discontinuities. In line with that, in the sympatric crested newts (*Triturus cristatus*), landscape factors such as forest gravel roads and south/south-west facing slopes contributed substantially to genetic differentiation between populations together with geographic distance (Haugen et al., 2020). In the palmate newt, *L. helveticus*, car traffic on secondary roads was found to select for short-legged newts due to a higher mortality of more mobile individuals characterised by long hind limbs, thus hind limb length, and consequently animals’ dispersal ability, changed according to a landscape characteristic in the studied subpopulations (Trochet et al., 2016). In our study, both the permutation tests and the clustering analysis indicated that genetic distinction between two local regions, although roads did not separate them specifically. This finding, together with the lack of correlation between the observed distribution of genetic variation and geographic distance between ponds, implies that natural landscape parameters that were not taken into account here may have important roles in shaping genetic differentiation patterns in smooth newts as well. Previous studies have successfully integrated the identification of influential landscape characteristics into their investigations of connectivity between populations in various amphibian species (e.g., Atlas & Fu, 2019; Lenhardt et al., 2017; Haugen et al., 2020). We propose that a similar approach should also be adopted by future population genetics studies on this caudate species, as both natural barriers and human-induced environmental changes may affect first and most severely those occasional migrants that connect distant breeding populations, ultimately leading to hindered gene flow and strong genetic structure at relatively small geographical scales.

ACKNOWLEDGEMENTS

We are indebted to the Pilisi Parkerdő Zrt. for allowing us to use their forestry roads. The study reported in this paper complies with current laws on animal

experimentation in Hungary and the European Union, and was approved by the institutional ethics committee (Centre for Agricultural Research, Plant Protection Institute, Institutional Animal Care and Use Committee; ATK NÖVI MÁB) in accordance with Good Scientific Practice guidelines and national legislation. All sampling procedures and experimental manipulations of this study were reviewed and specifically approved by the national authority of the Middle-Danube-Valley Inspectorate for Environmental Protection, Nature Conservation and Water Management, who issued the permission to capture, handle and collect samples from the animals (KTF: 603-3/2014, KTF: 603-4/2014, KTF: 2771-3/2015). Map data copyrighted OpenStreetMap contributors and available from <https://www.openstreetmap.org>.

Funding

Z. Tóth was financially supported by the Postdoctoral Research Program of the Hungarian Academy of Sciences (MTA, SZ-029/2013), the Prémium Postdoctoral Research Programme of the Hungarian Academy of Sciences (MTA, PREMIUM-2018-198) and the National Research, Development and Innovation Office (NKFIH, PD108938). E. Nemesházi was financially supported by the Austrian Agency for International Cooperation in Education & Research (OeAD-GmbH; ICM-2019-13228, Ernst Mach Grant worldwide).

REFERENCES

Almeida-Gomes, M. & Rocha, C.F.D. (2014). Landscape connectivity may explain anuran species distribution in an Atlantic forest fragmented area. *Landscape Ecology* 29, 29–40. Doi: 10.1007/s10980-013-9898-5

Archer, F.I., Adams, P.E. & Schneiders, B.B. (2017). stratag: An R package for manipulating, summarizing and analysing population genetic data. *Molecular Ecology Resources* 17, 5–11. Doi: 10.1111/1755-0998.12559

Arendt, J. D. (2015). Effects of dispersal plasticity on population divergence and speciation. *Heredity* 115, 306–311. Doi: 10.1038/hdy.2015.21

Arntzen, J.W., Kuzmin, S., Beebee, T., Papenfuss, T., Sparreboom, M., Ugurtas, I.H. et al. (2009). *Lissotriton vulgaris*. IUCN Red List of Threatened Species 2009: e.T59481A11932252. [www.iucnredlist.org](http://www.iucnredlist.org). Downloaded on 17 July 2020.

Arntzen, J.W., Abrahams, C., Meilink, W.R., Iosif, R. & Zuiderwijk, A. (2017). Amphibian decline, pond loss and reduced population connectivity under agricultural intensification over a 38 year period. *Biodiversity and Conservation* 26(6), 1411–1430. Doi: 10.1007/s10531-017-1307-y

Atlas, J.E. & Fu, J. (2019). Isolation by resistance analysis reveals major barrier effect imposed by the Tsinling Mountains on the Chinese wood frog. *Journal of Zoology* 309, 69–75. Doi: 10.1111/jzo.12702

Bell, G. (1977). The life of the smooth newt (*Triturus vulgaris*) after metamorphosis. *Ecological Monographs* 47, 279–299. Doi: 10.2307/1942518

Billerman, S.M., Jesmer, B.R., Watts, A.G., Schlichting, P.E., Fortin, M.J., Funk, W.C., Hapeman, P., Muths, E. & Murphy, M.A. (2019). Testing theoretical metapopulation conditions with genotypic data from Boreal Chorus Frogs (*Pseudacris*

*maculata*). *Canadian Journal of Zoology* 97, 1042–1053. Doi: 10.1139/cjz-2018-0275

Bird, C.E., Karl, S.A., Smouse, P.E. & Toonen, R.J. (2011). Detecting and measuring genetic differentiation. In: *Crustacean Issues: Phylogeography and Population Genetics in Crustacea*, pp. 31–55. Koenemann, S., Held, C., Schubart, C., Eds., Boca Raton, CRC Press. Doi: 10.1201/b11113-4

Blanquart, F., Gandon, S. & Nuismer, S. L. (2012). The effects of migration and drift on local adaptation to a heterogeneous environment. *Journal of Evolutionary Biology* 25, 1351–1363. Doi: 10.1111/j.1420-9101.2012.02524.x.

Bókony, V., Móricz, Á. M., Tóth, Z., Gál, Z., Kurali, A., Mikó, Z., Pásztor, K., Szederkényi, M., Tóth, Z., Ujszegi, J., Üveges, B., Krüzselyi, D., Capon, R.J., Hoi, H. & Hettyey, A. (2016). Variation in chemical defense among natural populations of common toad, *Bufo bufo*, tadpoles: the role of environmental factors. *Journal of Chemical Ecology* 42, 329–338. Doi: 10.1007/s10886-016-0690-2

Buono, V., Galliani, G., Mancini, E., Davoli, F., Mengoni, C., Mucci, N. & Vignoli, L. (2018). An improved microsatellite panel to assess genetic variability of the Italian smooth newt (*Lissotriton vulgaris meridionalis*). *Journal of Genetics* 97, 569–573. Doi: 10.1007/s12041-018-0934-8

Caballero, A., & García-Dorado, A. (2013). Allelic diversity and its implications for the rate of adaptation. *Genetics* 195, 1373–1384. Doi: 10.1534/genetics.113.158410

Chao, A.N., Chiu, C.H. & Jost, L. (2014). Unifying species diversity, phylogenetic diversity, functional diversity, and related similarity and differentiation measures through Hill numbers. *Annual Review of Ecology, Evolution, and Systematics* 45, 297–324. Doi: 10.1146/annurev-ecolsys-120213-091540

De Meeûs, T., & Goudet, J. (2007). A step-by-step tutorial to use HierFstat to analyse populations hierarchically structured at multiple levels. *Infection, Genetics and Evolution* 7, 731–735. Doi: 10.1016/j.meegid.2007.07.005

Diniz-Filho, J. A. F., Soares, T. N., Lima, J. S., Dobrovolski, R., Landeiro, V. L., Telles, M. P. D. C., Rangel, T. F. & Bini, L. M. (2013). Mantel test in population genetics. *Genetics and Molecular Biology* 36, 475–485. Doi: 10.1590/S1415-47572013000400002

Dolmen, D. (1981). Local migration, rheotaxis and philopatry by *Triturus vulgaris* within a locality in central Norway. *British Journal of Herpetology* 6, 151–158.

Emel, S.L., Olson, D.H., Knowles, L.L. & Storfer, A. (2019). Comparative landscape genetics of two endemic torrent salamander species, *Rhyacotriton kezeri* and *R. variegatus*: implications for forest management and species conservation. *Conservation Genetics* 20, 801–815. Doi: 10.1007/s10592-019-01172-6

Frankham, R., Ballou, J.D. & Briscoe, D.A. (2010). *Introduction to Conservation Genetics*, 2nd Edition. Cambridge, UK, Cambridge University Press.

Gaggiotti, O.E., Chao, A., Peres-Neto, P., Chiu, C.-H., Edwards, C., Fortin, M.-J., Jost, L., Richards, C.M. & Selkoe, K.A. (2018). Diversity from genes to ecosystems: a unifying framework to study variation across biological metrics and scales. *Evolutionary Applications* 11, 1176–1193. Doi: 10.1111/eva.12593

Goldberg, C.S., Kaplan, M.E. & Schwalbe, C.R. (2003). From the frog's mouth: buccal swabs for collection of DNA from

- amphibians. *Herpetological Review* 34, 220–221.
- Goudet, J. & Jombart, T. (2015). hierfstat: Estimation and Tests of Hierarchical F-Statistics. R package version 0.04-22. <https://CRAN.R-project.org/package=hierfstat>
- Goudet, J., Raymond, M., de Meeüs, T. & Rousset, F. (1996). Testing differentiation in diploid populations. *Genetics* 144, 1933–1940.
- Goudet, J. (2005). Hierfstat, a package for R to compute and test hierarchical F-statistics. *Molecular Ecology Notes* 5, 184–186. Doi: 10.1111/j.1471-8286.2004.00828.x
- Griffiths, R.A. (1984). Seasonal behaviour and intrahabitat movements in an urban population of smooth newts, *Triturus vulgaris* (Amphibia: Salamandridae). *Journal of Zoology* 203, 241–251. Doi: 10.1111/j.1469-7998.1984.tb02330.x
- Guillot, G., Estoup, A., Mortier, F., & Cosson, J.F. (2005). A spatial statistical model for landscape genetics. *Genetics* 170(3), 1261–1280. Doi: 10.1534/genetics.104.033803
- Guillot, G., Renaud, S., Ledevin, R., Michaux, J., & Claude, J. (2012). A unifying model for the analysis of phenotypic, genetic, and geographic data. *Systematic Biology* 61(6), 897–911. Doi: 10.1093/sysbio/sys038
- Haugen, H., Linløkken, A., Østbye, K. & Heggenes, J. (2020). Landscape genetics of northern crested newt *Triturus cristatus* populations in a contrasting natural and human-impacted boreal forest. *Conservation Genetics* 21, 515–530. Doi: 10.1007/s10592-020-01266-6
- Hoffmann, A.A. & Willi, Y. (2008). Detecting genetic responses to environmental change. *Nature Reviews Genetics* 9, 421–432. Doi: 10.1038/nrg2339
- Holderegger, R., Kamm, U., & Gugerli, F. (2006). Adaptive vs. neutral genetic diversity: implications for landscape genetics. *Landscape Ecology* 21, 797–807. Doi: 10.1007/s10980-005-5245-9
- Johanet, A., Picard, D., Garner, T.W., Dawson, D.A., Morales-Hojas, R., Jehle, R., Peltier, D. & Lemaire, C. (2009). Characterization of microsatellite loci in two closely related *Lissotriton* newt species. *Conservation Genetics* 10, 1903. Doi: 10.1007/s10592-009-9850-z
- Jost, L. (2006). Entropy and diversity. *Oikos* 113, 363–375. Doi: 10.1111/j.2006.0030-1299.14714.x
- Jost, L. (2008). GST and its relatives do not measure differentiation. *Molecular Ecology* 17, 4015–4026. Doi: 10.1111/j.1365-294X.2008.03887.x
- Jost, L. (2007). Partitioning diversity into independent alpha and beta components. *Ecology* 88, 2427–2439. Doi: 10.1890/06-1736.1
- Jost, L., Archer, F., Flanagan, S., Gaggiotti, O., Hoban, S. & Latch, E. (2018). Differentiation measures for conservation genetics. *Evolutionary Applications* 11, 1139–1148. Doi: 10.1111/eva.12590
- Lande, R. (1996). Statistics and partitioning of species diversity, and similarity among multiple communities. *Oikos* 76, 5–13.
- Lenhardt, P.P., Brühl, C.A., Leeb, C. & Theissinger, K. (2017). Amphibian population genetics in agricultural landscapes: does viniculture drive the population structuring of the European common frog (*Rana temporaria*)? *PeerJ* 5, e3520. Doi: 10.7717/peerj.3520
- Lesbarrères, D., Primmer, C.R., Lodé, T. & Merilä, J. (2006). The effects of 20 years of highway presence on the genetic structure of *Rana dalmatina* populations. *Ecoscience* 13, 531–538. Doi: 10.2980/1195-6860(2006)13[531:TEOYOH]2.0.CO;2
- Luqman, H., Muller, R., Vaupel, A., Brodbeck, S., Bolliger, J. & Gugerli, F. (2018). No distinct barrier effects of highways and a wide river on the genetic structure of the Alpine newt (*Ichthyosaura alpestris*) in densely settled landscapes. *Conservation Genetics* 19, 673–685. Doi: 10.1007/s10592-018-1046-y
- Marsh, D.M. & Trenham, P.C. (2001). Metapopulation dynamics and amphibian conservation. *Conservation Biology* 15, 40–49. Doi: 10.1111/j.1523-1739.2001.00129.x
- Martin, M., Théry, M., Rodgers, G., Goven, D., Sourice, S., Mège, P. & Secondi, J. (2016). UV wavelengths experienced during development affect larval newt visual sensitivity and predation efficiency. *Biology Letters* 12, 20150954. Doi: 10.1098/rsbl.2015.0954
- Matschiner, M. & Salzburger, W. (2009). TANDEM: integrating automated allele binning into genetics and genomics workflows. *Bioinformatics* 25, 1982–1983. Doi: 10.1093/bioinformatics/btp303
- Meirmans, P.G. (2006). Using the AMOVA framework to estimate a standardized genetic differentiation measure. *Evolution* 60(11), 2399–2402. Doi: 10.1111/j.0014-3820.2006.tb01874.x
- Meirmans, P. G. (2012). The trouble with isolation by distance. *Molecular Ecology* 21, 2839–2846. Doi: 10.1111/j.1365-294X.2012.05578.x
- Müllner, A. (2001). Spatial patterns of migrating great crested newts and smooth newts: the importance of the terrestrial habitat surrounding the breeding pond. *Rana* 4, 279–293.
- Nadachowska, K., Flis, I. & Babik, W. (2010). Characterization of microsatellite loci in the Carpathian newt (*Lissotriton montandoni*). *The Herpetological Journal* 20, 107–110.
- Nadachowska-Brzyska, K., Zieliński, P., Radwan, J. & Babik, W. (2012). Interspecific hybridization increases MHC class II diversity in two sister species of newts. *Molecular Ecology* 21, 887–906. Doi: 10.1111/j.1365-294X.2011.05347.x
- Niedzicka, M. E., Glowacki, B. M., Zieliński, P. & Babik, W. (2020). Morphology is a poor predictor of interspecific admixture—the case of two naturally hybridizing newts *Lissotriton montandoni* and *Lissotriton vulgaris* (Caudata: Salamandridae). *Amphibia-Reptilia* 1, 1–12. doi: 10.1163/15685381-bja10019
- OpenStreetMap contributors (2015). Planet dump [Data file from 16.07.2020]. Retrieved from <https://planet.openstreetmap.org>.
- Peakall, R.O.D. & Smouse, P.E. (2006). GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* 6, 288–295. Doi: 10.1111/j.1471-8286.2005.01155.x
- Petit, R.J., El Mousadik, A. & Pons, O. (1998). Identifying populations for conservation on the basis of genetic markers. *Conservation Biology* 12, 844–855. Doi: 10.1111/j.1523-1739.1998.96489.x
- Phipson, B. & Smyth, G.K. (2010). Permutation P-values should never be zero: calculating exact P-values when permutations are randomly drawn. *Statistical Applications in Genetics and Molecular Biology* 9, 39. Doi: 10.2202/1544-6115.1585
- Pidancier, N., Miquel, C. & Miaud, C. (2003). Buccal swabs as a non-destructive tissue sampling method for DNA analysis in amphibians. *The Herpetological Journal* 13, 175–178.
- Powers, R.P. & Jetz, W. (2019). Global habitat loss and extinction risk of terrestrial vertebrates under future land-use-change scenarios. *Nature Climate Change* 9(4), 323–329. Doi: 10.1038/s41558-019-0406-z
- RCore Team (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <https://www.R-project.org/>.
- Rousset, F. (2008). genepop’007: a complete re-implementation of the genepop software for Windows and Linux. *Molecular Ecology Resources* 8, 103–106. Doi: 10.1111/j.1471-8286.2007.01931.x
- Ryman, N., Palm, S., André, C., Carvalho, G.R., Dahlgren, T.G., Jorde, P.E., Laikre, L., Larsson, L.C., Palmé, A. & Ruzzante, D.E. (2006). Power for detecting genetic divergence: differences between statistical methods and marker loci. *Molecular Ecology* 15, 2031–2045. Doi: 10.1111/j.1365-294X.2006.02839.x
- Schmidt, P., Weddelling, K., Thomas, M., Rottschmidt, R., Tarkhnishvili, D.N. & Hachtel, M. (2006). Dispersal of *Triturus alpestris* and *T. vulgaris* in agricultural landscapes—comparing estimates from allozyme markers and capture-mark-recapture analysis. In: *Herpetologica Bonnensis* II. pp. 139–143. Vences, M., Köhler, J., Ziegler, T., Böhme, W., Eds., Bonn, Societas Europaea Herpetologica. Doi:
- Secondi, J. & Théry, M. (2014). An ultraviolet signal generates a conflict between sexual selection and species recognition in a newt. *Behavioral Ecology and Sociobiology* 68, 1049–1058. Doi: 10.1007/s00265-014-1717-8
- Secondi, J., Rodgers, G., Bayle, F., Sourice, S. & Théry, M. (2015). Mate preference, species recognition and multimodal communication in heterogeneous environments. *Evolutionary Ecology* 29, 217–227. Doi: 10.1007/s10682-014-9744-5
- Sinsch, U. & Kirst, C. (2016). Homeward orientation of displaced newts (*Triturus cristatus*, *Lissotriton vulgaris*) is restricted to the range of routine movements. *Ethology Ecology & Evolution* 28(3), 312–328. Doi: 10.1080/03949370.2015.1059893
- Smith, M.A. & Green, D.M. (2005). Dispersal and the metapopulation paradigm in amphibian ecology and conservation: are all amphibian populations metapopulations? *Ecography* 28, 110–128. Doi: 10.1111/j.0906-7590.2005.04042.x
- Smouse, P.E. & Peakall, R. (1999). Spatial autocorrelation analysis of individual multiallele and multilocus genetic structure. *Heredity* 82, 561–573. Doi: 10.1038/sj.hdy.6885180
- Sotiropoulos, K., Eleftherakos, K., Tsaparis, D., Kasapidis, P., Giokas, S., Legakis, A. & Kotoulas, G. (2013). Fine scale spatial genetic structure of two syntopic newts across a network of ponds: implications for conservation. *Conservation Genetics* 14, 385–400. Doi: 10.1007/s10592-013-0452-4
- Tóth, Z. (2015). Context-dependent plastic response during egg-laying in a widespread newt species. *PLoS ONE* 10, e0136044. Doi: 10.1371/journal.pone.0136044
- Tóth, Z. & Hettyey, A. (2018). Egg-laying environment modulates offspring responses to predation risk in an amphibian. *Journal of Evolutionary Biology* 31, 710–721. Doi: 10.1111/jeb.13258
- Tóth, Z., Hoi, H. & Hettyey, A. (2011). Intraspecific variation in the egg-wrapping behaviour of female smooth newts, *Lissotriton vulgaris*. *Amphibia-Reptilia* 32, 77–82. Doi: 10.1163/017353710X543001
- Van Oosterhout, C., Hutchinson, W.F., Wills, D.P. & Shipley, P. (2004). MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes* 4, 535–538. Doi: 10.1111/j.1471-8286.2004.00684.x
- Warwick, T. (1949). The colonization of bomb-crater ponds at Marlow, Buckinghamshire. *Journal of Animal Ecology* 18, 137–141.
- Watts, A.G., Schlichting, P., Billerman, S., Jesmer, B., Micheletti, S., Fortin, M.J., Funk, W.C., Hapeman, P., Muths, E. & Murphy, M.A. (2015). How spatio-temporal habitat connectivity affects amphibian genetic structure. *Frontiers in Genetics* 6, 275. Doi: 10.3389/fgene.2015.00275
- Weddelling, K., Hachtel, M., Sander, U. & Tarkhnishvili, D. (2004). Bias in estimation of newt population size: a field study at five ponds using drift fences, pitfalls and funnel traps. *The Herpetological Journal* 14, 1–8.
- Yang, R.C. (1998). Estimating hierarchical F-statistics. *Evolution* 52, 950–956. Doi: 10.1111/j.1558-5646.1998.tb01824.x
- Zieliński, P., Nadachowska-Brzyska, K., Wielstra, B., Szkotak, R., Covaciu-Marcov, S. D., Cogălniceanu, D. & Babik, W. (2013). No evidence for nuclear introgression despite complete mtDNA replacement in the Carpathian newt (*Lissotriton montandoni*). *Molecular Ecology* 22, 1884–1903. Doi: 10.1111/mec.12225
- Zieliński, P., Dudek, K., Arntzen, J. W., Palomar, G., Niedzicka, M., Fijarczyk, A., Liana, M., Cogălniceanu, D. & Babik, W. (2019). Differential introgression across newt hybrid zones: Evidence from replicated transects. *Molecular Ecology* 28, 4811–4824. Doi: 10.1111/mec.15251

Accepted: 1 December 2020

Please note that the Supplementary Materials are available via the Herpetological Journal website: <https://thebhs.org/publications/the-herpetological-journal/volume-31-number2-april-2021>





# Thermoregulation comparisons between a threatened native and an invasive lizard species

Yole Caruso<sup>1</sup>, Daniele Macale<sup>2</sup>, Luca Luiselli<sup>3,4,5</sup> & Leonardo Vignoli<sup>1</sup>

<sup>1</sup> Dipartimento di Scienze, Università Roma Tre, Viale Marconi, 446, 00146, Rome, Italy

<sup>2</sup> Fondazione Bioparco di Roma, Viale del Giardino Zoologico, 1, 00197, Rome, Italy

<sup>3</sup> IDECC, Institute for Development, Ecology, Conservation and Cooperation, via G. Tomasi di Lampedusa 33, 00144, Rome, Italy

<sup>4</sup> Department of Applied and Environmental Biology, Rivers State University of Science and Technology, P.M.B. 5080 Nkpolu, Port Harcourt, Rivers State, Nigeria

<sup>5</sup> University of Lomé, Faculty of Sciences, Department of Zoology, BP: 6057 Lomé, Togo

Lizard thermoregulation is costly and is largely behavioural. *Podarcis raffonei*, endemic of few islets of the Aeolian archipelago (southern Italy), is one of the most threatened lizards in Europe, its survival being under threat also due to the presence of the congeneric *P. siculus*, a successful invader characterised by behavioural plasticity and effectiveness and precision at regulating body temperature (T<sub>b</sub>). We tested whether thermoregulation behaviour diverges between the two species by analysing (i) the heating rates under a standard thermal condition, and (ii) the temperature at which lizards ended basking (T<sub>final</sub>) along a thermal gradient. Overall, we found behavioural differences between the two lizards (i.e. *P. siculus* exhibited lower T<sub>final</sub>), although both species had comparable heating rates and thermoregulated in the same thermal conditions. The invasive *P. siculus* had lower T<sub>final</sub> and, since heating rates were similar between species, it expended less time basking than native *P. raffonei*. We speculate that the observed thermal ecology differences could provide a selective advantage to *P. siculus* in the harsh island environment.

**Keywords:** *Podarcis raffonei*, *Podarcis siculus*, lizard, biological invasion, competition

## INTRODUCTION

Ectothermic organisms, such as reptiles, regulate their body temperature (T<sub>b</sub>), in relation to the external environmental temperatures, within a restricted range close to their physiological optimum (Cowles & Bogert, 1944; Shine & Kearney, 2001; Seebacher, 2005). The extent of the thermoregulation process varies from species to species, within populations, and among different environments, along a continuum from a wide to a narrow thermal range (e.g. Ruibal & Philibosian, 1970; Adolph, 1990; Hertz, 1992) also in relation to climate (e.g. Shine & Madsen, 1996) and microclimate (e.g. Paci et al., 2018). Thermoregulation is largely behavioural in reptiles, including lizards (Spellerberg, 1972; Castilla et al., 1999), and the maintenance of optimal physiological performances is unequivocally habitat-selection-dependent (e.g. Huey, 1991; Reinert, 1993). For instance, body temperature (T<sub>b</sub>) regulation in lacertid lizards is controlled by (i) adapting activity patterns to the external temperature range, (ii) selecting thermally favourable micro-habitats (e.g., basking spots) and (iii) using postural adjustments that alter the exchange of heat with the environment (Bauwens & Herts, 1996).

Interspecific competitors can temporarily disturb the achievement of optimal T<sub>b</sub> by pushing away from

or exploiting optimal basking places, thus leading to a reduction in performance by direct competition or interference (Cady & Joly, 2003; Luiselli, 2008; Žagar et al., 2015).

In Europe, where the thermal ecology of lizards has been generally intensely studied (e.g. Ortega & Martín-Vallejo, 2019), several threatened species do occur in small Mediterranean islands (IUCN, 2020). In these arid islands, lizard populations generally face extreme scarcity of trophic resources and individual thermoregulation can be affected by strong temporal changes in both abiotic and biotic conditions, thus limiting the time available to forage (see Pérez-Mellado, 1989; Castilla & Bauwens, 1991; Lo Cascio, 2006; Capula & Lo Cascio, 2011; Capula et al., 2014). The IUCN's (2020) Critically Endangered Aeolian lizard, *Podarcis raffonei* (Mertens, 1952), endemic of the Aeolian archipelago in southern Italy (Capula et al., 2002), is the most threatened lizard in Europe (Capula, 2004; Capula & Lo Cascio, 2006, 2011). The continued survival of this species is uncertain due not only to the very small range (Capula et al., 2002), but also to the presence of the congeneric Italian wall lizard *P. siculus* (Rafinesque-Schmaltz, 1810) (Capula et al., 2002), an invasive lacertid accidentally introduced in historical time in the Aeolian Archipelago. It has been hypothesised that *P. siculus* can negatively impact

native lizards, including *P. raffonei*, through competition, displacement and hybridisation (Capula et al., 2002). The overall colonising success of *P. siculus* is considered to be due to quick acclimatisation and adaptability to new environmental conditions propensities, dispersal abilities (Deichsel et al., 2010; Vignoli et al., 2012), and likely to effectiveness and precision at regulating its T<sub>b</sub> (Kapsalas et al., 2016; Ortega et al., 2016). Although the constraints of the thermal environment may affect ectotherm species distribution and population density, they have been rarely considered jointly with the possible outcome of interspecific competition, i.e. between invader and native species (Angert et al., 2002).

Preliminary data on field T<sub>b</sub> of *Podarcis raffonei* were published by Lo Cascio (2006) and Capula et al. (2014), but up to now the thermo-physiological and thermo-ecological characteristics of this species versus its potential competitor (*P. siculus*) have remained largely unexplored. In addition, no study is available on the thermal ecology of these species under identical environmental quality conditions.

In the present study, by two experiments in controlled arenas, we analyse the thermoregulation characteristics of the two species within the same range of available temperatures. In previous studies, *P. siculus* were more exploratory, bold and neophilic than the sympatric native congeneric lizards (Damas-Moreira et al., 2019). Therefore, it can be hypothesised that *P. siculus* may also differ from *P. raffonei* in thermoregulation performance. Specifically, we tested in comparative experiments (i) the heating rate under standard thermal conditions, and (ii) the temperature at which lizards ended basking (T<sub>final</sub>) along a thermal gradient. We expect that the possible competitive advantage of *P. siculus* on *P. raffonei* should be, among other factors (Mencía et al., 2017), in thermoregulation traits (Angilletta, 2009) rather than in anatomical adaptations (Camacho et al., 2015). That is: (i) the two congeneric species do not differ in the rate of heating rates due to morphological and anatomical similarities (Gvoždík, 2002), and (ii) *P. siculus* is more efficient than *P. raffonei* in some traits of the thermal biology (i.e. faster achievement of and/or lower T<sub>b</sub> to enter activity).

## MATERIALS AND METHODS

### Study species

The Aeolian lizard *Podarcis raffonei* is a small lizard, up to 7.5 cm SVL, that inhabits harsh environments (for instance, in Strombolicchio and La Canna islets), with rocky walls facing the sea and very sparse vegetation. In Vulcano, *P. raffonei* inhabits a human-altered area and some small promontories (Corti & Lo Cascio, 1999). The Italian wall lizard *P. siculus* is a medium-sized (up to 9 cm SVL, Corti et al., 2010) species that ranges throughout Italy south of the Alps, including many islands in the Tyrrhenian Sea, and along the Adriatic coastal area to Montenegro. It is also an invasive species established in several countries outside its native range (Crnobrnja-Isailovic et al., 2009).

### Protocol

Lizards were collected from two areas: we sampled *P. raffonei* individuals in May 2017, at the Capogrosso area in the island of Vulcano (Sicily; 38°25'6.98"N, 14°56'32.80"E). For logistical reasons we were unable to catch *P. siculus* individuals from the Aeolian Archipelago. However, since this species is extremely widespread, ecologically generalist and even introduced in different regions of the world, we collected individuals of this species in the same location where *P. raffonei* was hosted (Rome, Latium; 41°54'59.99"N, 133 12°29'16.77"E) also to avoid translocation of an invasive species. The Aeolian lizard population was collected for an ex-situ conservation project headed by the Department of Sciences – Roma Tre University and the Fondazione Bioparco di Roma. After the capture, the individuals were transported to the facilities of the Reptile House at the Fondazione Bioparco di Roma, formerly known as the municipal zoo in Rome. Capture, captive keeping and authorisation for performing studies were given by the Ministry of the Environment and the Protection of the Territory and the Sea (note 0008937; May 2nd, 2017). Sex was determined by the presence/absence of active femoral pores (present in males) and by the width and shape of the head (larger in males). All lizards were adults, females were not pregnant, and each of them was measured for the snout-vent-length (SVL, mm). All individuals were housed in enclosures (1.0 x 1.0 x 0.9 m), with sand as substrate, pine bark as refuges and density set at 5 individuals/m<sup>2</sup>.

### Experiment I – Accumulation of heat

In order to analyse whether there were any differences between *P. raffonei* and *P. siculus* regarding the heating rates, we impeded lizard individuals to adopt any behavioural compensatory mechanism while being tested (Gvoždík, 2002) by placing them into a fauna-box (10 cm x 15 cm), filled with river sand as a substrate, that was positioned within a larger storage box. Above the box an infrared heating lamp (Philips 230-50V) was positioned vertically, supported using a tripod. We measured temperature at the ground inside the fauna-box in five points (four vertices and the centre) by using a digital laser infrared thermometer (±0.1 °C; Seafront AT380) set for the measurement of sandy substrates. The reduced box size allowed obtaining a homogeneous temperature (32 ± 0.3 °C; mean ± SD) within the box to test thermoregulation efficiency of lizards under the same conditions of radiation. The duration of the heat exposure was 10 minutes. The set of temperatures used in our experiment did not exceed 33 °C, in order to not exceed the maximum of the average T<sub>b</sub> recorded in the field for *P. raffonei* individuals at Faraglione and at Strombolicchio islets (respectively, 34.7 °C and 33.2 °C) (Lo Cascio, 2006, 2010). For each tested individual, the body temperature at the start of the experiment (T<sub>initial</sub>) was measured by inserting a K-type thermocouple probe (0.5 mm in diameter) connected to a digital microprocessor thermometer (HD9218, Delta Ohm, Italy) 5 mm into its cloaca (Gvoždík, 2002). After T<sub>initial</sub> recording, the individual was introduced into the fauna box.



During the 10 minutes of test, the cloacal temperature was measured 10 times, once per minute. The time taken to take the individual and measure the cloacal temperature (average = 15 seconds) was homogeneous for the various measurements and among the various individuals, thus it did not bias the thermoregulation process and measurements. After measuring the cloacal temperature, the individual was quickly repositioned in the box. After the 10 minutes of testing, the final cloacal temperature ( $T_{final}$ ) was measured. We defined as  $\Delta T$  the difference between  $T_{final}$  and  $T_{initial}$ . No individual that was housed in the experiments died or lost its tail, thus indicating that the welfare of the captive animals was adequately maintained.

Experiment II - Thermoregulation behaviour

In order to evaluate differences in the performance of thermoregulation and to evaluate any difference between the two species as for the behavioural regulation of body temperature in thermally heterogeneous habitats (e.g. basking duration, choice of the basking site), experimental arenas were built with a gradient of basking temperatures that could be chosen independently by individuals during the test. Seven individuals were randomly selected for each species and sex. This small sample size was due to the extreme rarity and threatened status of *P. raffonei*. The arena was prepared inside a climatized room with temperatures ranging 15-17 °C. The ambient temperature was selected to have almost inactive animals to be tested. Inside the room, we placed four 1 m x 1 m boxes to house the animals (divided by species and sex) as well as the experimental boxes within which to conduct the tests. The setting of the experimental boxes was dictated by the need to make a basking area that would provide a temperature gradient with a single > 30 °C spot not straightforwardly available to lizards (i.e. raised above the ground). This hot spot was made by placing a piece of cork (10 cm height and 5 cm<sup>2</sup> in upper surface) under a spot lamp (Sera Reptil Alu Reflector 200; diameter 200 mm) carrying a bulb (Solar raptor UV HID-lamp-70 W spot-beam) (Fig. 1). The basking area generated by the lamp heat consisted of a gradient of decreasing temperatures the further away from the hot spot. The temperature gradient was subdivided into eight radial sectors out of which six were evenly spaced (by 5 cm), representing distinct basking sites associated with different temperatures (Fig. 1). Temperature at the ground of the various basking areas was measured by a digital laser infrared thermometer (0.1 °C; Seafrost AT380) set for the measurement of sandy substrates both before each experiment and after two hours from turning on the lamps to allow them to reach steady temperature. On top of the cork the temperature was 31.0 °C, 7 °C higher than at the ground (sector 1) (Fig. 1). We set 31.0 °C as maximum temperature following the same reasons as in experiment I. Before starting each experiment, the air temperatures of the room and the experimental arena were recorded. Furthermore, before starting the test, the cloacal temperature ( $T_{initial}$ ) of each lizard was also measured.

The beginning of the experiment started by

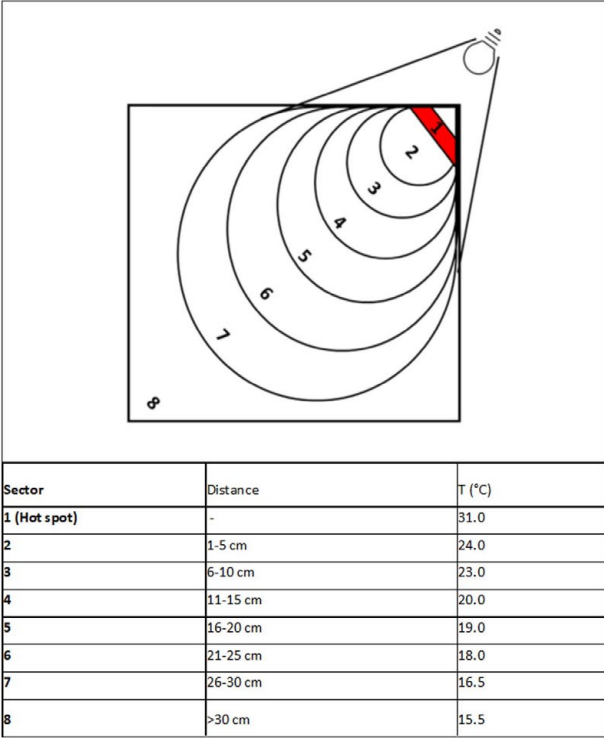


Figure 1. Arrangement of the experimental arena. Relative distance from the hot spot (1) and temperature at ground (°C) for each sector (2-8) are also indicated.

positioning the animal in the furthest point opposite the hot spot (sector 8, at room temperature; Fig. 1). All the various movements and the time spent by the lizards across sectors were recorded. The test ended when the animal left the thermoregulation area and moved to sector 8 for at least one minute. The total duration of the experiment was recorded (total time, seconds). At the end of each test, cloacal temperatures ( $T_{final}$ ) were recorded. All the experiments were video-recorded with the experimenters being not visible to the lizards. The experiments were conducted between 10:00 AM and 03:00 PM (Rome standard time) and involved two persons (YC and LV).

Statistical analyses

We built several General and Generalised linear models to test the effect of species, sex, SVL, and basking site on the thermoregulation behaviour of the lizards. To test the rate of heat accumulation, we conducted a GLM (Repeated Measures ANOVA) with the cloacal temperature measured at 1-minute intervals as response variable, species and sex as fixed factors, and the 10 repeated measures of cloacal temperature as the treatment. We tested the effects of (1) species and sex on the basking site (defined as hot spot vs. remaining sectors of the thermal gradient) choice (binomial error distribution and link logit function; dependent variable: basking site; fixed factors: species and sex; covariate:  $T_{final}$ ); (2) species, sex, basking site on  $T_{final}$  (normal error distribution; dependent variable:  $T_{final}$ ; fixed factors: species and sex; covariates: SVL,  $T_{initial}$ ); (3) species, sex, basking site on  $\Delta T$  (normal error distribution; dependent variable:  $\Delta T$ ;

Table 1. Results of the Repeated Measures ANOVA conducted on the rate of accumulation of heat by lizards. The effects of species and sex (group variables) on the cloacal temperature measured at 1-minute intervals (response variable) are shown. R1=treatment (10 repeated measures of cloacal temperature).

Effect	SS	DoF	MS	F	P
Intercept	182862.8	1	182862.8	8121.005	0
Species	21.5	1	21.5	0.953	0.341
Sex	126.1	1	126.1	5.599	0.028
Species*Sex	2.1	1	2.1	0.094	0.762
Error	427.8	19	22.5		
R1	3302.4	10	330.2	261.428	<0.001
R1*species	6.5	10	0.6	0.514	0.879
R1*sex	5.9	10	0.6	0.47	0.908
R1* Species*Sex	5.5	10	0.5	0.434	0.928
Error	240	190	1.3		

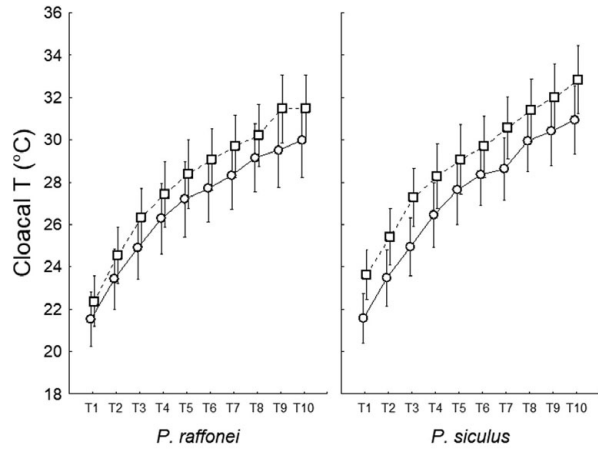


Figure 2. Experiment on accumulation of heat. Cloacal temperature measured at 1-minute intervals for 10 minutes on males (squares) and females (circles) of *P. raffonei* and *P. siculus*. Vertical bars denote 95 % confidence intervals.

fixed factors: species and sex; covariates: SVL, “total time”,  $T_{initial}$ ); (4) species, sex, basking site on “total time” (normal error distribution; dependent variable: “total time”; fixed factors: species and sex; covariate: SVL). For each model, we estimated the overall fit by means of regression between sum of squares of the total model vs. residuals (multiple  $R^2$ ; for the General Linear Models) and of ratio between degree of freedom and scaled deviance (DoF/SDev; for the Generalised Linear Models). Models with high multiple  $R^2$  or ratio DoF/SDev around 1 were considered as satisfactorily explaining all (random) error variability in the data (i.e. no overdispersion) (Nelder & Wedderburn, 1972). All the models provided a good fit to the data but model (4) that showed a marginal lack of fit (see Supplementary Material). All tests were carried out by using Statistica v 8.0 (Statsoft) with two tails and alpha set at 0.05.

RESULTS

Experiment I – Accumulation of heat

The data referring to the individuals tested for the accumulation of heat experiment are summarised in Table S1. There was no significant effect for any of the

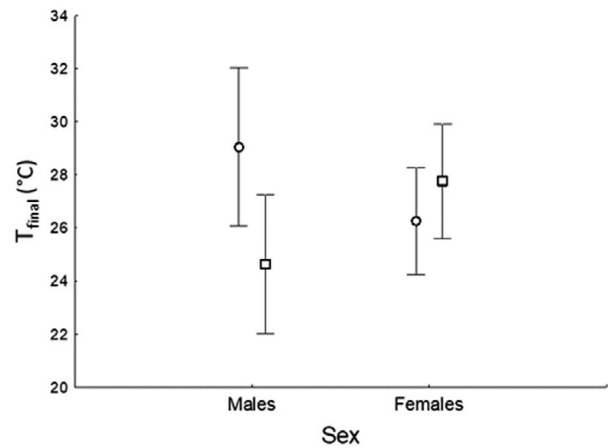
predictors tested in the Repeated Measures ANOVA model except for the sex. The temperatures reached through the one-minute-step did not vary significantly between the two species (Table 1; Fig. 2). Female *Tb* was significantly higher than males in the first 3-minutes of treatment before slowing down reaching the same trend as males for the rest of the treatment ( $F_{1,19} = 5.60$ ,  $P = 0.028$ ). On the other hand,  $\Delta T$  was not influenced by sex, species, or interaction species\*sex (for all tests,  $F \leq 0.865$  and  $p \geq 0.360$ ). Similarly, the  $T_{final}$  was not influenced by any of the considered factors and covariates (for all tests,  $F \leq 1.670$  and  $p \geq 0.208$ ).

Experiment II - Thermoregulation behaviour

A first GLM model showed that species, sexes and their interaction did not influence the choice of the basking site (for all effects,  $Wald \leq 1.663$ ;  $p \geq 0.197$ ). As for  $T_{final}$ , only the basking site ( $F_{1,19} = 14.390$ ,  $p < 0.001$ ) and the interaction term species\*sex ( $F_{1,19} = 5.328$ ,  $p = 0.032$ ) showed an effect (Table S2). Individuals that used the hot spot showed higher  $T_{final}$  ( $29.9 \pm 2.3$  °C) than lizard that thermoregulated in the remaining sectors ( $24.8 \pm 2.4$  °C). Moreover, *P. siculus* males had  $T_{final}$  lower ( $26.36 \pm 2.71$  °C) than *P. raffonei* males ( $29.33 \pm 2.20$  °C) regardless of body size (SVL) and  $T_{initial}$  (Post-hoc test:  $p < 0.01$ ; Fig. 3). There was a positive effect of the basking site on  $\Delta T$  ( $F_{1,18} = 13.132$ ,  $p = 0.001$ ), with individuals basking on the hot spot showing a greater  $\Delta T$  than those basking elsewhere (Table S3). There was a positive correlation between  $\Delta T$  and  $T_{initial}$  ( $F_{1,18} = 20.711$ ,  $p < 0.001$ ), that is the lower the  $T_{initial}$  the more the difference with  $T_{final}$ . No effect of species, sex, SVL, and ‘total time’ on  $\Delta T$  was detected (for all tests  $F \leq 3.121$  and  $p \geq 0.094$ ), whereas a positive effect of the species\*sex interaction term was observed ( $F_{1,18} = 6.769$ ,  $p = 0.018$ ), with  $\Delta T$  being significantly different between the two species: male *P. siculus* individuals accumulated lower  $\Delta T$  than male *P. raffonei*. The ‘total time’ of the experiment duration was significantly different between the sexes (Table S4), with females of both species spending less time basking than males ( $F_{1,20} = 6.055$ ,  $p = 0.023$ ).

DISCUSSION

Our study consolidates knowledge on how *P. siculus*



**Figure 3.** Experiment of thermal behaviour. Effect of species\*sex interaction on T<sub>final</sub> (circles=*P. raffonei*; squares=*P. siculus*). Vertical bars denote 95 % confidence intervals.

appears to be a precise (Van Damme et al., 1990) and accurate (Kapsalas et al., 2016) thermoregulator, and also reveals the ability to enter activity at lower Tb than *P. raffonei*. These features may not represent direct advantages in interspecific competition. However, it is possible that, by improving the overall effectiveness of thermoregulation, *P. siculus* may have advantages to overcome the thermal challenges of new and/or changing environments (Kapsalas et al., 2016).

As expected, the two study species thermoregulate actively by basking, and the time spent at basking may be considerable for lizards (Avery, 1976). Therefore, lizards that spent less time in basking may invest more time in other activities (for instance, foraging and mating). Thus, minimising the basking time would convey selective advantages in terms of both fitness and survival (for instance, by reducing predation risk due to minimised exposure time). Biophysical models predict that the time spent by lizards in basking may be reduced through physiological or behavioural adjustments of two traits: set-point temperature (= preferred Tb) and heating rate (Bakken & Gates, 1975). Although we did not estimate the preferred Tb in a laboratory photothermal gradient (Gvoždík, 2002), we obtained the temperature at which lizards stop basking in laboratory conditions (T<sub>final</sub>). T<sub>final</sub> is therefore proportional to the time spent in basking by a given species, all other things being equal. In our case, *P. siculus* should be advantaged as its T<sub>final</sub> was lower than that of *P. raffonei*. Instead, our lizards of both species showed comparable heating rates and thermoregulated in the same thermal conditions. Therefore, we expected that T<sub>final</sub> contribute the most to reduce basking time (Huey & Slatkin, 1976).

Accumulation of heat

As expected, the temperatures reached at each one-minute step did not vary significantly between the two species indicating that the two species accumulate heat in the same way and do not present significant differences in accumulating body heat (Gvoždík, 2002). The observed intersexual difference is likely due to the sexual size dimorphism in *Podarcis* species (Kaliontzopoulou et al.,

2007), with males significantly larger than females. In fact, a smaller body accumulates heat at a faster rate, all other factors being equal (Avery, 1976).

Thermoregulation behaviour

Males of *P. siculus* enter full activity at a lower body temperature than males of *P. raffonei*. This finding would suggest that the lower T<sub>final</sub> observed in *P. siculus* may confer a selective advantage over *P. raffonei* during the daily routine activities. The highest T<sub>final</sub> was observed in those individuals that thermoregulated in the sector 1 (hot spot), regardless of species and gender. That is, under experimental conditions, *P. siculus* and *P. raffonei* did not show apparent difference in the selection of the hotspot. We also found that *P. siculus* did not thermoregulate faster than *P. raffonei*. Instead, female thermoregulation was shorter than in males irrespective of species. This finding supports the evidence obtained through the experiment on heating rates. The lack of interspecific divergence in basking duration seems to be counterintuitive given the lower Tb that was needed by *P. siculus* to end basking. We speculate that, since thermal quality of the basking site (i.e. hot spot vs. remaining sectors) influenced T<sub>final</sub>, the expected shorter basking time by male *P. siculus* may have been blurred by stochastic choice of basking site. This hypothesis is partly supported by the fact that *P. siculus* males had a higher variance of T<sub>final</sub> than those of *P. raffonei*, thus indirectly revealing a higher heterogeneity in basking site (= sector) selection. In nature, where optimal and suboptimal basking sites are likely equally accessible, the advantage of a lower Tb needed to end basking and entering full activity is expected to also confer a faster thermoregulation process (Kapsalas et al., 2016).

In conclusion, our study pointed out that there are subtle differences in the basking quality and correlated behaviours between an invasive lizard and a threatened endemic species, with potential implications for coexistence dynamics. Obviously, it cannot be excluded that the observed thermoregulation differences between the species may be non-relevant for the interspecific competition outcome. Other factors are possibly involved in giving *P. siculus* a potentially competitive advantage over the native species, like diet strategy, aggressive behaviour, or running speed mediated by T<sub>final</sub>. These and other factors are likely to influence overall lizard behaviour and to contribute to the outcome of interspecific interactions. We would urge researchers to perform further field and mesocosm long-term studies on the thermal ecology of these two species, in order to verify whether the observed interspecific differences may influence the respective fitness of the coexisting populations.

REFERENCES

Adolph, S.C. (1990). Influence of behavioral thermoregulation on microhabitat use by two Sceloporus lizards. *Ecology* 71, 315–327.

Angert, A.L., Hutchison, D., Glossip, D. & Losos, J.B. (2002). Microhabitat use and thermal biology of the collared

lizard (*Crotaphytus collaris collaris*) and the fence lizard (*Sceloporus undulatus hyacinthinus*) in Missouri glades. *Journal of Herpetology* 36, 23-29.

Angilletta, M.J. (2009). *Thermal Adaptation: A Theoretical and Empirical Synthesis*. Oxford, UK: Oxford University Press.

Avery, R.A. (1976). Thermoregulation, metabolism and social behaviour in Lacertidae. In, *Morphology and Biology of Reptiles*. Eds. A.d'A. Bellairs & C.B. Cox. Pp. 245-259. London, UK: Academic Press.

Bakken, G.S. & Gates, D.M. (1975). Heat-transfer analysis of animals: some implications for field ecology, physiology, and evolution. *Perspectives of Biophysical Ecology*. Pp. 255-290. Springer, Berlin, Heidelberg, DE.

Bauwens, D. & Herts, P. (1996). Thermoregulation in a Lacertid Lizard: The Relative Contributions of Distinct Behavioral Mechanisms. *Ecology* 77(6), 1818.

Cady, A. & Joly, P. (2003). Competition for basking places between the endangered European pond turtle (*Emys orbicularis galloitalica*) and the introduced red-eared slider (*Trachemys scripta elegans*). *Canadian Journal of Zoology* 81(8), 1392-1398.

Camacho, A., Pavao, R., Moreira, C.N., Pinto, A.C.B.F., Navas, C.A. & Rodrigues, M.T. (2015). Interaction of morphology, thermal physiology and burrowing performance during the evolution of fossoriality in Gymnophthalmi lizards. *Functional Ecology* 29(4), 515-521.

Capula, M. (2004). Low genetic variation in a critically endangered Mediterranean lizard: conservation concerns for *Podarcis raffonei* (Reptilia, Lacertidae). *Italian Journal of Zoology* 71(1), 161-166.

Capula, M., Luiselli, L., Bologna, M.A. & Ceccarelli, A. (2002). The decline of Aeolian wall lizard, *Podarcis raffonei*: causes and conservation proposals. *Oryx* 36(1), 66-72.

Capula, M. & Lo Cascio, P. (2006). *Podarcis raffonei* (Mertens, 1952). In, *Atlante degli Anfibi e dei Rettili d'Italia/Atlas of Italian Amphibians and Reptiles*. Pp. 480-485. Sindaco, R., Doria, G., Razzetti, E. & Bernini, F. (Eds.), Polistampa. Firenze, IT.

Capula, M. & Lo Cascio, P. (2011). *Podarcis raffonei* (Mertens, 1952). In, *Fauna d'Italia*. Vol XLV, Reptilia Pp. 401-410. Corti, C., Capula, M., Luiselli, L., Razzetti, E. & Sindaco, R. (Eds.), Calderini Il Sole 24 ORE, Bologna, IT.

Capula, M., Corti, C., Lo Cascio, P. & Luiselli, L. (2014). Thermal ecology of the Aeolian wall lizard, *Podarcis raffonei*. What about body temperatures in microinsular lizards? In, *Scripta Herpetologica*. Studies on Amphibians and Reptiles in honour of Benedetto Lanza. Capula, M. & Corti, C. (Eds.). Belvedere, Latina, IT.

Castilla, A.M. & Bauwens, D. (1991). Thermal biology, microhabitat selection, and conservation of the insular lizard *Podarcis hispanica atrata*. *Oecologia* 85, 366-374.

Castilla, A.M., Van Damme, R. & Bauwens, D. (1999). Field body temperatures, mechanisms of thermoregulation and evolution of thermal characteristics in lacertid lizards. *Natura Croatica* 8(3), 253-274.

Corti, C. & Lo Cascio, P. (1999). I lacertidi italiani. L'Epos, Palermo, IT.

Corti, C., Capula, M., Luiselli, L., Razzetti, E. & Sindaco, R. (2010). Reptilia. Fauna d' Italia Vol. XIV. Calderini Il Sole24 Ore, Milano, IT.

Cowles, R.B. & Bogert, C.M. (1944). A preliminary study of the

thermal requirements of desert reptiles. *Bulletin of the American Museum of Natural History* 83, 265-296.

Crnobrnja-Isailovic, J., Vogrin, M., Corti, C., Pérez Mellado, V., Sá-Sousa, P., Cheylan, M., Pleguezuelos, J., Sindaco, R., Romano, A. & Avci, A. (2009). *Podarcis siculus* (incorrect version published in 2016). The IUCN Red List of Threatened Species 2009: e.T61553A86151752.

Damas-Moreira, I., Riley, J.L., Harris, D.J. & Whiting, M.J. (2019). Can behaviour explain invasion success? A comparison between sympatric invasive and native lizards. *Animal Behaviour* 151, 195-202.

Deichsel, G., Nafis, G. & Hakim, J. (2010). Geographic distribution: *Podarcis siculus*. *Herpetological Review* 41, 513-514.

Gvoždík L. (2002). To heat or to save time? Thermoregulation in the lizard *Zootoca vivipara* (Squamata: Lacertidae) in different thermal environments along an altitudinal gradient. *Canadian Journal Zoology* 80(3), 479-492.

Hertz, P.E. (1992). Temperature regulation in Puerto Rican Anolis lizards: a field test using null hypotheses. *Ecology* 73, 1405–1417.

Huey, R.B. (1991). Physiological consequences of habitat selection. *The American Naturalist* 137, S91-S115.

Huey, R.B. & Slatkin, M. (1976). Cost and benefits of lizard thermoregulation. *The Quartely Review of Biology* 51(3), 363-384.

IUCN (2020). The IUCN Red List of Threatened Species. Available at: [www.iucnredlist.org](http://www.iucnredlist.org). Accessed on May 11th, 2020.

Kaliontzopoulou, A., Carretero, M.A. & Llorente, G.A. (2007). Multivariate and geometric morphometrics in the analysis of sexual dimorphism variation in Podarcis lizards. *Journal of Morphology* 268(2), 152-165.

Kapsalas, G., Gavriilidi, I., Adamopoulou, C., Foufopoulos, J. & Pafilis, P. (2016). Effective thermoregulation in a newly established population of *Podarcis siculus* in Greece: a possible advantage for a successful invader. *Acta Herpetologica* 11(2), 111-118.

Lo Cascio, P. (2006). Aspetti ecologici e problemi di conservazione di una popolazione di *Podarcis raffonei* (Reptilia: Lacertidae). *Naturalista Siciliano* 30(3-4), 463-489.

Lo Cascio, P. (2010). Attuali conoscenze e misure di conservazione per le popolazioni relitte dell'endemica lucertola delle Eolie, *Podarcis raffonei*. *Naturalista Siciliano* 34(3-4), 295-317.

Luiselli, L. (2008). Resource partitioning in freshwater turtle communities: a null model meta-analysis of available data. *Acta Oecologica* 34(1), 80-88.

Mencia, A., Ortega, Z., & Pérez-Mellado, V. (2017). From tameness to wariness: chemical recognition of snake predators by lizards in a Mediterranean island. *PeerJ* 5 e2828.

Mertens, R. (1952). Neue Eidechsenrassen von der Liparischen Inseln. *Senckenbergiana* 32(56), 309-314.

Nelder, J. A., & Wedderburn, R. W. (1972). Generalized linear models. *Journal of the Royal Statistical Society: Series A (General)* 135(3), 370-384.

Ortega, Z., Mencia, A., & Pérez-Mellado, V. (2016). Thermal ecology of *Podarcis siculus* (Rafinesque-Schmalz, 1810) in Menorca (Balearic Islands, Spain). *Acta Herpetologica* 11(2), 127-133.

Ortega, Z., & Martín-Vallejo, F. J. (2019). Main factors affecting lacertid lizard thermal ecology. *Integrative Zoology* 14(3), 293-305.



- Paci, O., Akani, G.C., Eniang, E.A., Segniagbeto, G.H., Nasone, W.R., Luiselli, L. & Vignoli, L. (2018). Who does bask longer? A comparison between elapid and viperid snakes in the field and in experimental conditions. *Zoologischer Anzeiger* 277, 116-120.
- Pérez Mellado, V. (1989). Estudio ecológico de la lagartija balear *Podarcis lilfordi* (Günther, 1874) en Menorca. *Revista de Menorca* 53, 455-511.
- Reinert, H.K. (1993). Habitat selection in snakes. In, *Snakes, Ecology and Behavior*. Pp. 201-240. Seigel, R.A. & Collins, J.T. (Eds.), McGraw-Hill, New York, NY.
- Rugiero, L. (1994). Food habits of the Ruin Lizard, *Podarcis sicula* (Rafinesque-Schmaltz, 1810), from a coastal dune in Central Italy. *Herpetozoa* 7(1/2), 71-73.
- Ruibal, R. & Philibosian, R. (1970). Eurythermy and niche expansion in lizards. *Copeia*, 645-653.
- Seebacher, F. (2005). A review of thermoregulation and physiological performance in reptiles: what is the role of phenotypic flexibility? *Journal of Comparative Physiology* B 175(7), 453-461.
- Shine, R. & Kearney, M. (2001). Field studies of reptile thermoregulation: how well do physical models predict operative temperatures? *Functional Ecology* 15(2), 282-288.

- Shine, R. & Madsen, T. (1996). Is thermoregulation unimportant for most reptiles? An example using water pythons (*Liasis fuscus*) in tropical Australia. *Physiological Zoology* 69(2), 252-269.
- Spellerberg, I.F. (1972). Temperature tolerances of southeast Australian reptiles examined in relation to reptile thermoregulatory behaviour and distribution. *Oecologia* 9(1), 23-46.
- Van Damme, R., Bauwens, D., Castilla, A.M. & Verheyen, R.F. (1990). Comparative thermal ecology of the sympatric lizards *Podarcis tiliguerta* and *Podarcis sicula*. *Acta Oecologia* 11, 503-512.
- Vignoli, L., Vuerich, V. & Bologna, M.A. (2012). Experimental study of dispersal behaviour in a wall lizard species (*Podarcis sicula*) (Sauria Lacertidae). *Ethology Ecology and Evolution* 24(3), 244-256.
- Žagar, A., Carretero, M.A., Osojnik, N., Sillero, N. & Vrezec, A. (2015). A place in the sun: interspecific interference affects thermoregulation in coexisting lizards. *Behavioral Ecology Sociobiology* 69(7), 1127-1137.

Accepted: 5 December 2020

Please note that the Supplementary Materials are available via the Herpetological Journal website:  
<https://thebhs.org/publications/the-herpetological-journal/volume-31-number2-april-2021>



Published by the British  
Herpetological Society

## Occurrence of lizards in agricultural land and implications for conservation

Marta Biaggini<sup>1</sup> & Claudia Corti<sup>1</sup>

<sup>1</sup> Museo di Storia Naturale, Università degli Studi di Firenze, Sede “La Specola”, Via Romana 17, 50125 Firenze, Italy

Agriculture intensification is among one of the major threats affecting terrestrial reptiles worldwide. There is however a lack of information available on the ecology of these vertebrates in agricultural landscapes. Basic information like the pattern of occurrence in cultivated fields is key to assess the probability of an animal being affected by threats driven by agricultural managing. Focussing on the Italian wall lizard (*Podarcis siculus*), we performed a field study to assess in detail its distribution and abundance in two cultivations, vineyards and cereal fields. Lizard distribution and abundance significantly varied among land uses, regardless of the arthropod fauna composition and diversity (analysed in the same fields), and the management activities. In the cereal fields, lizards were present exclusively along the field margins while in the vineyards they also occurred in the inner portions of the cultivated areas, even if they were more abundant next to the borders. The widespread presence of lizards in the vineyards suggest that *P. siculus* can likely adapt to such cultivated areas. This partly lowers the effect of habitat loss due to vineyard planting but exposes animals to the risks related to management activities, including mechanical practices and chemical application. In contrast, the presence of sowed lands, as extremely simplified habitats, results primarily in a definitive loss of habitat for lizards that are unable to settle within them, while the exposure to threats driven by management is less direct than in vineyards.

**Keywords:** agriculture, *Podarcis*, reptiles, wildlife protection

## INTRODUCTION

Agricultural intensification has widely transformed the traditional agricultural landscapes throughout the European countries, including the Mediterranean region, typically replacing complex and heterogeneous landscapes with simplified and depleted ones (Benton et al., 2003; Tscharntke et al., 2005). Intensively cultivated areas have rapidly expanded to the detriment of patches of natural and semi-natural vegetation, small croplands and ecotones, resulting in habitat loss, landscape uniformisation and becoming the leading cause of biodiversity loss in agroecosystems (Sala et al., 2000).

Agriculture intensification and intensive use of natural resources are among the most common threats affecting terrestrial reptiles worldwide (Gibbons et al., 2000; Todd et al., 2010; Böhm et al., 2013). These vertebrates, usually have relatively small home ranges and a limited dispersal ability (Huey, 1982), thus being directly exposed to the effects of changes in land use and agricultural management. The presence of reptiles (and especially lizards) can play an important ecological role in agro-environments. The diet of these vertebrates, being mainly insectivorous and less often showing specialisation, allows them to survive and attain relatively

high densities also in depleted ecosystems (Regal, 1983), as cultivated lands can be, thus providing an important food resource for higher level predators. Consequently, changes in their population densities can have cascading effects on other trophic levels over the long-term (Martín & López, 1996; Díaz et al., 2006). With this perspective in mind, analysing and monitoring lizard distribution and density in agricultural habitats can be of particular interest for wildlife conservation. There is a general lack of information available on the distribution and ecology of reptiles in agricultural landscapes, especially at field scale (but see Wisler et al., 2008; Biaggini et al., 2009; Amaral et al., 2012a; Biaggini & Corti, 2015; Biaggini & Corti, 2017), as well as on the effects of agriculture management on these vertebrates (Driscoll, 2004; Berry et al., 2005). Analysing the occurrence of a species inside agricultural habitats is essential to assess its risk of exposure to possible threats driven by management, such as mechanical activities or a chemicals' application. Indeed, the probability of animals to be exposed to a certain threat mainly depends on the overlap (in time and space) between their presence and the appearance of the threat in the fields (Ockleford et al., 2018).

In this study we analysed, at the field-scale, lizard distribution and abundance inside different agricultural

Correspondence: Marta Biaggini (marta.biaggini@virgilio.it)

land uses, focussing on the Italian wall lizard, *Podarcis siculus* (Rafinesque-Schmaltz, 1810). This species is quite widespread inside agricultural landscapes and, at least to some extent, it is able to adapt to and take advantage of human-altered environments (Biaggini & Corti, 2015; Biaggini & Corti, 2017). In particular, we wanted to determine the actual occurrence of lizards in two cultivations common to central Italy, vineyards and cereal fields, verifying if (and how far) lizards are present inside the cultivated patches or if they occur just along the field boundaries. Moreover, in order to test if food availability could be a driver shaping lizard distribution in the two land uses, we also analysed the arthropod fauna composition and diversity in the same fields.

## METHODS

### Study species

*Podarcis siculus* is a medium sized lacertid lizard mainly distributed in Italy, in most of the surrounding islands, and along the eastern coast of the Adriatic Sea. In Central Italy, where the study was performed, *P. siculus* concentrates its annual activity between early spring and late autumn, usually occurs at low elevation, and in open habitats (Corti et al., 2010). When compared with syntopic species, *P. siculus* shows a preference for relatively arid vs. humid microhabitats, both on rocky surfaces and open meadows, avoiding tree cover (Van Damme et al., 1990; Capula et al., 1993).

### Study area

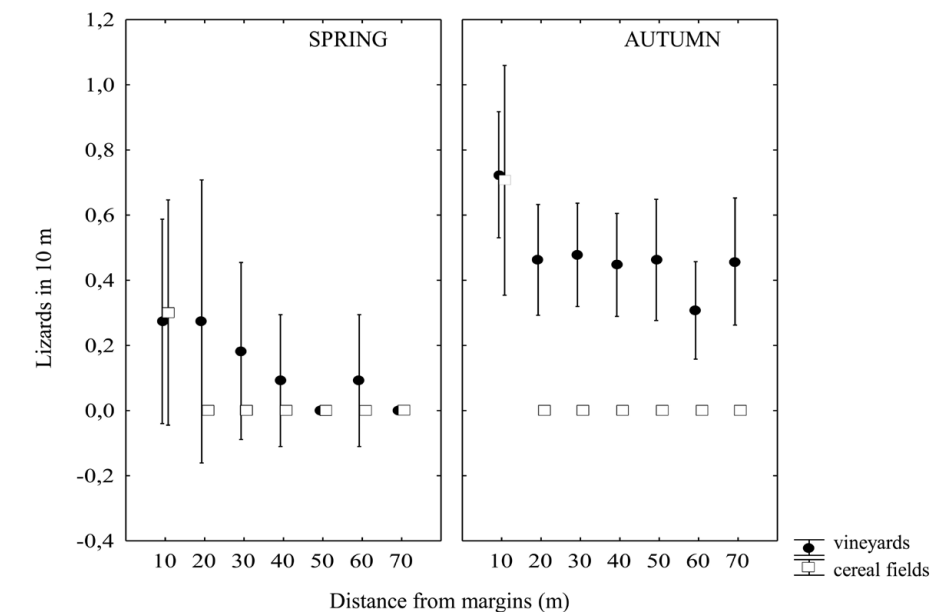
The study was performed in an agricultural area in central Italy (43°40' N, 11°09' E, total extension = about 280 ha; elevation = 90–150 m a.s.l.; annual range of temperature = -0.1–35.9 °C; annual precipitation = 620.80 mm), in four vineyards and two cereal fields (Fig. 1). We choose sites lying on the same slope in order to reduce the variability of environmental factors like sun and wind exposure. This choice limited the number of cereal fields available for samplings. On the other hand, due to the irregular shape of the vineyards, for this land use we had to include more than two sites in order to have a sufficient sample of transect segment far from the field margins (> 50 m, see Statistical analyses). Vineyards were characterised by conventional management, including use of chemical compounds and mechanical management activities, while in the cereal fields (that were sowed in the autumn preceding our study) no agricultural practices, including harvest, were performed during our sampling period due to a wildlife management program.

### Sampling procedure

In order to record lizard abundance, we performed 103 linear transects (length ranging from 100 to 380 m) covering the surfaces of the four vineyards (76 transects) and the two cereal fields (27 transects). Considering that *Podarcis* lizards usually run distances of less than 3 m while escaping from potential predators (e.g., Rugiero, 1997; Diego-Rasilla, 2003; Biaggini et al., 2009), within



**Figure 1.** Examples of the surveyed land uses: cereal fields (above) and vineyards (below) during field activity, in May (left) and September (right).



**Figure 2.** Mean number of lizards observed in 10 m transects' segments at increasing distances from uncultivated field margins, inside vineyards (black circles) and cereal fields (white squares), in spring and autumn.

each site, transects were parallel and more than 15 m far apart one to the other, to prevent multiple recordings of the same individual. Transects were walked in May and September – October 2006, on sunny days between 7:00 and 19:00 h, covering the whole daily activity period of *Podarcis siculus* (Foà et al., 1992). In the same sites we sampled epigeal arthropods, using 28 pitfall traps randomly distributed (16 in the cereal fields, 12 in the vineyards), filled with a solution of vinegar and acetylsalicylic acid. Traps were emptied and replaced once every 14 days from April – July (five sampling periods). This method is particularly suitable for collecting ground-dwelling arthropods (Biaggini et al., 2007) that represent the majority of the diet of *P. siculus* (Pérez-Mellado & Corti, 1993), thus providing information on the potential food availability. All arthropods were identified to the level of order; after identification, specimens were oven dried at 70 °C and for each trap, the content of the single samplings was weighed to the nearest 0.0001 g. In order to further verify a possible correlation between lizard and arthropod fauna presence, we collected data on *P. siculus* density and arthropod fauna diversity in agricultural lands, from papers listed in Table 1 and related databases. All data were gathered with the same techniques used for the present study.

### Statistical analyses

We divided each transect into 10 m long segments (667 segments in total) and for each segment we extrapolated the number of lizards observed. In order to analyse lizard abundance, we performed a Generalised Linear Model (GLM) using the number of lizards per segment as the dependent variable (not normally distributed even after log-transformation, Kolmogorov-Smirnov:  $n = 667$ ,  $d = 0.415$ ,  $P < 0.01$ ), land use (vineyards vs cereal fields), distance from the nearest uncultivated margin (from 0

– 10 m to 60 – 70 m) and season (spring vs autumn) as fixed factors.

In order to characterise the superficial arthropod fauna of the study sites, we analysed faunal composition, biodiversity (using the Shannon-Wiener index calculated on arthropod orders,  $H$ ) and dry weight (as a proxy of biomass). We assessed the pattern of faunal composition among the 28 traps by performing a Principal Component Analysis (PCA) on the relative proportions of arthropods' orders, calculated on the total number of specimens (we arcsine transformed the proportions to avoid the complications present in analysing compositional data). We analysed arthropod diversity and biomass through GLM analyses, using  $H$  (Kolmogorov-Smirnov:  $d = 0.064$ ,  $p = n.s.$ ) and the dry weight (Kolmogorov-Smirnov:  $d = 0.068$ ,  $p = n.s.$ ) of single samplings as dependent variables, land use (vineyards vs cereal fields) and distance from the field margin (near if  $< 10$  m; far if  $> 10$  m) as fixed factors. In these analyses we used the 10 m distance from the nearest margin to classify traps as near or far from the border, corresponding to the transect segment giving the strongest results for lizard density. With the aim of further investigating the possible correlation between lizard abundance and arthropod diversity, we performed a GLM on the reference data listed in Table 1, considering lizard density as dependent variable,  $H$  index calculated on arthropod orders and season as continuous and categorical predictors, respectively. We used STATISTICA software for all the analyses (StatSoft, Inc., 2011).

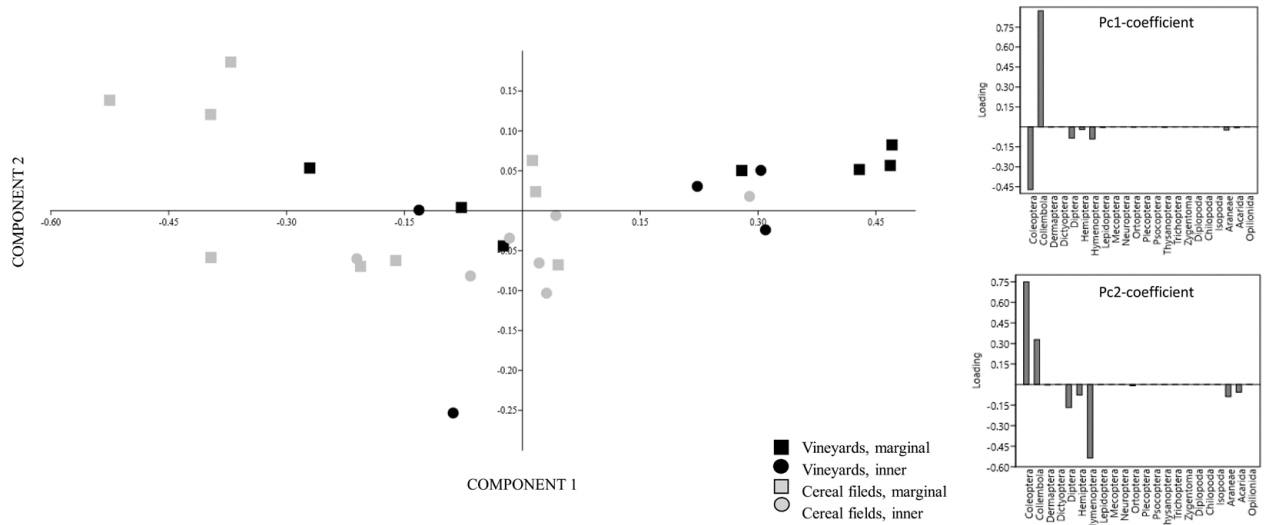
## RESULTS

The number of lizards observed in 10 m long segments with increasing distance from the margins towards the inside of fields significantly differed among land uses, distances from field margins, seasons and in relation



**Table 1.** Mean values (± st. dev.) of density of *Podarcis siculus* (N in 100 m) and Shannon-Wiener index of arthropod orders (H) in several agricultural land uses in Italy (months of samplings are indicated in brackets; n.a. = not available). Data were extrapolated from reference papers and related databases (Ref.: present study = p.s.; Biaggini & Corti, 2015 = a; Biaggini et al., 2015a = b; Biaggini et al., 2015b = c; Corti et al., 2015 = d). In italics: data not used in the analyses, here reported to give account of the range of *P. siculus* density in the considered land uses.

Land use	Area, n. of sites	N in 100 m (months)	H (months)	Ref.
Unmanaged olive grove	Sardinia, 1	0 (V-VI); 0.889 ± 1.018 (X)	2.094 ± 0.227 (V-VI); 1.502 ± 0.326 (X)	d
Traditional olive grove	<i>S Tuscany, 6</i>	<i>0.806 ± 0.554 (V-VI)</i>	<i>n.a.</i>	<i>a</i>
	Sardinia, 6	0.889 ± 0.565 (III-IV)	2.004 ± 0.369 (III-IV)	b
	Sardinia, 1	3.704 ± 2.313 (V-VI); 9.259 ± 5.481 (X)	2.402 ± 0.404 (V-VI); 2.081 ± 0.217 (X)	d
Conventional olive grove	<i>S Tuscany, 3</i>	<i>0.077 ± 0.277 (V-VI)</i>	<i>n.a.</i>	<i>a</i>
	Sardinia, 3	0.704 ± 0.539 (III-IV)	2.050 ± 0.384 (III-IV)	b
	Sardinia, 1	1.748 ± 0.780 (V-VI); 2.747 ± 0.780 (X)	1.840 ± 0.592 (V-VI); 1.863 ± 0.114 (X)	d
Vineyard	<i>S Tuscany, 3</i>	<i>0.438 ± 0.729 (V-VI)</i>	<i>n.a.</i>	<i>a</i>
	N Tuscany, 4	0.997 ± 0.671 (V); 2.569 ± 1.231 (IX-X)	1.576 ± 0.439 (IV-VI); n.a.	p.s.
Sowable land	<i>S Tuscany, 8</i>	<i>0.106 ± 0.550 (V-VI)</i>	<i>n.a.</i>	<i>a</i>
	N Tuscany, 1	0 (IV-VI); 0.079 ± 0.238 (X)	1.797 ± 0.587 (V-VI); 1.776 ± 0.923 (X-XI)	c
	Veneto, 1	0 (IV-VI); 0 (IX-X)	1.487 ± 0.477 (V-VI); 1.228 ± 0.359 (IX-X)	c
Set aside	N Tuscany, 1	0 (IV-VI); 0.180 ± 0.270 (X)	1.817 ± 0.167 (V-VI); 1.935 ± 0.352 (X-XI)	c
	Veneto, 1	0.048 ± 0.167 (IV-VI); 0 (IX-X)	1.480 ± 0.288 (V-VI); 1.151 ± 0.750 (IX-X)	c
Unmanaged sowable land	N Tuscany, 2	0.300 ± 0.483 (V); 0.702 ± 0.323 (IX-X)	2.132 ± 0.270 (IV-VI); n.a.	p.s.



**Figure 3.** Scatter plot displaying PCA performed on arthropod order composition of the pitfall traps put in vineyards and cereal fields (marginal and inner portions for both land uses are represented).

**Table 2.** Comparison of lizard density in 10 m transects’segments in relation to the distance from the nearest uncultivated margin (from 0-10 m to 60-70 m), the land use (vineyard vs cereal filed) and the season (spring vs autumn). The number of transects’segments (N) per comparison is shown.

	d.f.	Wald	P	N (tot = 667)
Intercept	1	1388.273	< 0.001	
Distance from margin (1)	6	206.691	< 0.001	10, 20, 30, 40, 50 m = 103; 60 m = 87; 70 m = 65
Land use (2)	1	345.177	< 0.001	Vineyards = 575; Cereal fields = 92
Season (3)	1	36.868	< 0.001	Spring = 140; Autumn = 527
(1)*(2)	6	104.473	< 0.001	
(1)*(3)	6	27.059	< 0.001	
(2)*(3)	1	30.331	< 0.001	
(1)*(2)*(3)	6	31.369	< 0.001	

to the interaction of the three variables (Table 2, Fig. 2). Vineyards hosted more lizards than cereal fields; for both land uses, we recorded the highest number of individuals in the first 10 m from the field margins and higher densities in autumn than in spring. In the cereal fields, we observed lizards exclusively in the first transect segments (0 – 10 m) and, in particular, within 3 m from the margins (personal observations).

We identified 22 orders of arthropods, among which Collembola, Coleoptera, Hymenoptera and Diptera represented 56.5 %, 19.7 %, 10.2 % and 7.1 % of the collected specimens, respectively. Traps from different land uses showed no striking differences in faunal composition; however, traps from the cereal fields were clustered slightly leftmost along the axis of the first principal component (explaining 85 % of variance), with higher relative abundance of Coleoptera and lower abundance of Collembola (Fig. 3). A GLM performed on arthropod order diversity revealed higher values of the Shannon-Wiener index in the unmanaged cereal fields than in the vineyards (n samplings: cereal fields = 74, vineyards = 56; F = 15.886, P < 0.001), and no significant differences between marginal and inner portions of the fields in both land uses (n samplings: margin = 65, inside = 65; F = 0.083, P = 0.774). The same analysis performed on the arthropod dry weight revealed no significant differences among land uses (F = 1.913, P = 0.169) and with varying distances from field margins (F = 0.313, P = 0.577). Lizard density did not vary in relation to arthropod diversity (n= 18, Wald = 0.121, P = 0.728) in spring and autumn (Wald = 0.965, P = 0.326) based on the reference data collected in Table 1.

DISCUSSION

Assessing the patterns of occurrence of a species is crucial to identify the major threats suffered by animals and the extent of their exposure to such threats within a certain habitat. In agricultural landscapes, where the

demand for effective conservation measures is pressing, such information is very scarce, especially for some vertebrates. We found substantial differences in the distribution of the Italian wall lizard among agricultural land uses. In the cereal fields, lizards were present exclusively in a narrow belt along the field margins while in the vineyards they also occurred in the inner portions of the cultivated areas, though with significantly lower densities than next to the borders (in the first 10 m). In autumn, we recorded the same pattern of distribution than in spring in both land uses, but with higher lizard densities due to the large presence of juveniles (accounting for about 62 % and 68 % of lizards inside cereal fields and vineyards, respectively).

The home range of *Podarcis siculus* covers up to 300 m² (Foà et al., 1990; Avery, 1993) and, consequently, most of lizards’ activities are probably concentrated in about a 10 m radius around the home range cores. Therefore, lizards recorded in the cereal fields, next to the borders, probably settle in the adjoining habitats. On the contrary, the occurrence of adults and juveniles (performing shorter movements; Braña, 2003) up to 70 m inside vineyards may entail a quite stable presence of lizards in this land use, at least during the warm season (when sampling was performed). The two crops were characterised by comparable arthropod fauna composition and biomass, while the unmanaged cereal fields (where less lizards occurred) hosted higher levels of arthropod biodiversity than vineyards. Considering that *P. siculus* feeds mainly on epigeal arthropods without remarkable specialisations (Pérez-Mellado & Corti, 1993; Rugiero, 1994; Burke & Mercuro, 2002), these observations suggest that food availability, at least as revealed by our analyses, was not the key feature explaining the striking differences in the presence of lizards found between the two types of cultivated lands. Farming disturbance, as well, was not probably among the main factors influencing lizard abundance and distribution. In fact, cereal fields were not managed during the study, while vineyards underwent the ordinary management, including machinery activity.

In accordance with Díaz & Carrascal (1991), who suggested that the structural requirements of habitats play a primary role in shaping lizard abundance, much greater than the role played by food availability, the very dissimilar habitat structure of cereal fields and vineyards could be a key factor influencing lizard presence in our study system. At small scales, the occurrence of lizards, as well as of different animal groups, in terms of species composition and relative abundance, strictly depends on the vegetation features and physical structure of habitats (Tews et al., 2004; Vitt et al., 2007; Mizsei et al., 2020). Sowed lands are extremely simplified habitats, characterised by only herbaceous vegetation (e.g., the cereal ears), bare soil and, consequently, by the almost complete lack of shade and shelters, except for quite deep vertical crevices when the soil dries, which may provide temporary refuges from predators. Vineyards, on the contrary, display a more complex structure, offering lizards different shelters (soil crevices, holes at the base of the vine trunks and support poles, vine leaves

and trunks) and shadow, when vines sprout (from the end of March). Such conditions in vineyards probably meet the conflicting needs imposed by thermoregulatory and anti-predatory requirements, allowing lizards to minimise the shuttling distance between sun (where they can bask) and the vegetation cover that provides both shade and the possibility of hiding (Carrascal & Díaz, 1989). Habitats that are more complex usually reduce the exposure to predation for lizards (Huey & Slatkin, 1976), among agricultural land uses as well (Biaggini et al., 2009). Analogously, the high density of lizards next to the margins of both land uses could be due to the complex structure of uncultivated boundaries in the study area, including shrubs and bushes, which are primarily important refuges for lizards (Strijbosch, 1988; Martín & López 1990; Martín & López 1998). Among cultivated lands, which are typically open habitats, the complexity of habitat structure allows higher abundance of reptiles in terms of both individuals and species (Biaggini & Corti, 2015). This pattern fits also if focussing on *P. siculus*, whose abundance inside agricultural lands follows a gradient of habitat complexity, higher in olive groves followed by vineyards and arable lands (Table 1).

Although further studies are needed to better understand the activity patterns of lizards within crops, our observations give some basic information on the distribution and abundance of these vertebrates in agricultural lands, which may have useful implications for conservation. From the pattern of presence of a species inside a crop, we can infer its exposure to different threats such as habitat loss or management activities (e.g., chemical spread). In vineyards, *P. siculus* can probably find environmental conditions favourable enough to settle there, partly compensating for the habitat loss due to vineyard planting through adaptation to the new environment. However, the stable presence of lizards in vineyards may entail a high and direct exposure to the risks related to management activities, including mechanical practices (e.g., mechanical grape harvesting and tillage) and chemical application. There are few studies exploring the possible impacts of management on lizard's populations, mostly focussing on pesticide application (e.g., Amaral et al., 2012a; Amaral et al., 2012b) and, at our knowledge, none of them deals with long-term effects. Moreover, in wild populations, complex interactions among ecological factors and human induced alterations occur, making it difficult to understand the mechanisms that, in some cultivated lands, allow lizards to cope with agricultural managing. Focussing on such mechanisms could be key in order to assess the treatment thresholds allowing lizard populations to persist in land uses such as vineyards. In contrast, following our observations, lizards do not settle in cereal fields but exploit only the marginal zones of these crops (a few meters besides the uncultivated margins), probably for feeding or basking, as observed for other reptiles (Wisler et al., 2008). Thus, the presence of sowable lands, as extremely simplified habitats, results primarily in a definitive loss of habitat for lizards. Given the low dispersal ability of these vertebrates, the maintenance of uncultivated habitats becomes key for the conservation of lizards in agricultural areas dominated by arable lands.

These observations further stress the negative impact that the expansion of huge monocultures has on the abundance and diversity of herpetofauna, along with the loss of those semi-natural landscape elements essential to maintain the connectivity in the unsuitable matrix of cultivated lands (Kleijn et al., 2011; Biaggini & Corti, 2015; Nopper et al., 2017). On the other hand, the field-scale analysis of lizard occurrence, suggests that in cereal fields the exposure to threats driven by management, such as chemical spread, is reasonably less direct than in vineyards. Consequently, the research effort to assess the risk for lizards to be exposed to pesticides in croplands should probably involve buffer habitats such as field margins, uncultivated patches, vegetated banks of rivers and ditches.

ACKNOWLEDGMENTS

We would like to thank S. Allain and S. Batista for their useful comments and suggestions; S. Perrone and F. Balò, “Azienda Agricola di Montepaldi” (University of Florence) for facilitating surveys in the study area.

REFERENCES

Amaral, M.J., Carretero, M.A., Bicho R.C., Soares, A.M.V.M & Mann, R.M. (2012a). The use of a lacertid lizard as a model for reptile ecotoxicology studies: Part 1 – Field demographics and morphology. *Chemosphere* 87, 757-764.

Amaral, M.J., Bicho R.C., Carretero, M.A., Sanchez-Hernandez, J.C., Faustino, A.M.R., Soares, A.M.V.M & Mann, R.M. (2012b). The use of a lacertid lizard as a model for reptile ecotoxicology studies: Part 2 – Biomarkers of exposure and toxicity among pesticide exposed lizards. *Chemosphere* 87, 765-774.

Avery, R.A. (1993). Diel variation in area of movement of the lizard *Podarcis siculus*. *Ethology Ecology & Evolution* 5, 511–518.

Benton, T.G., Vickery, J.A. & Wilson, J.D. (2003). Farmland biodiversity: is habitat heterogeneity the key? *Trends in Ecology & Evolution* 18, 182–8, doi: 10.1016/S0169-5347(03)00011-9.

Berry, O., Tocher, M.D., Gleeson, D.M. & Sarres, S.D. (2005). Effect of vegetation matrix on animal dispersal: genetic evidence from a study of endangered skinks. *Conservation Biology* 19, 855–864, doi: 10.1111/j.1523-1739.2005.00161.x.

Biaggini, M., Consorti, R., Dapporto, L., Dellacasa, M., Paggetti, E. & Corti, C. (2007). The taxonomic level order as a possible tool for rapid assessment of Arthropod diversity in agricultural landscapes. *Agriculture Ecosystems & Environment* 122, 183–191, doi: 10.1016/j.agee.2006.12.032.

Biaggini, M., Berti, R. & Corti, C. (2009). Different habitat, different pressures? Analysis of the escape behaviour and ectoparasite load in *Podarcis sicula* (Lacertidae) populations in different agricultural habitats. *Amphibia-Reptilia* 30, 453–461, doi: 10.1163/156853809789647068

Biaggini, M. & Corti, C. (2015). Reptile assemblages across agricultural landscapes: where does biodiversity hide? *Animal Biodiversity and Conservation* 38.2, 163–174.

Biaggini, M. & Corti, C. (2017). Variability of breeding resource partitioning in a lacertid lizard at field scale. *Animal Biology* 67, 81–92, doi: DOI: 10.1163/15707563-00002523.

Biaggini, M., Lo Cascio, P., Bassu, L., Bazzoffi, P., Barbagli, F., Nulchis, V. & Corti, C. (2015a). Ecological focus area – EFA: the biological value of olive groves. A case study in Sardinia (Italy) / Ecological focus area - EFA: il valore biologico degli oliveti. Un caso di studio in Sardegna. *Italian Journal of Agronomy* 10(s1), 748, doi: 10.4081/ija.2015.748.

Biaggini, M., Lo Cascio, P., Bazzoffi, P. & Corti, C. (2015b). Effectiveness of GAEC cross-compliance Standard 4.2c for biodiversity conservation in set-asides, part II (ground-dwelling Arthropods and Vertebrates) / Efficacia dello Standard BCAA di Condizionalità 4.2c per il mantenimento della biodiversità nei suoli ritirati dalla produzione, parte II (Artropodi epigei e Vertebrati). *Italian Journal of Agronomy* 10(s1), 699, doi: 10.4081/ija.2015.699.

Böhm, M., Collen, B., Baillie, J.E.M., Bowles, P., Chanson, J., Cox, N., Hammerson, G., Hoffmann, M., Livingstone, S.R., Ram, M., Rhodin, A.G.J., Stuart, S.N. et al. (2013). The Conservation Status of the World's Reptiles. *Biological Conservation* 157, 372-385, doi: 10.1016/j.biocon.2012.07.015.

Braña, F. (2003). Morphological correlates of burst speed and field movement patterns: the behavioural adjustment of locomotion in wall lizards (*Podarcis muralis*). *Biological Journal of the Linnean Society* 80, 135–146, doi: 10.1046/j.1095-8312.2003.00226.x.

Burke, R.L. & Mercuro, R.J. (2002). Food habits of a New York population of Italian Wall Lizard, *Podarcis siculus* (Reptilia, Lacertidae). *American Midland Naturalist* 147, 368–375, doi: 10.1674/0003-0031(2002)147[0368:FHOANY]2.0.CO;2.

Capula, M., Luiselli, L. & Rugiero, L. (1993). Comparative ecology in sympatric *Podarcis muralis* and *P. sicula* (Reptilia: Lacertidae) from the historical centre of Rome: what about competition and niche segregation in an urban habitat? *Italian Journal of Zoology* 60, 287–291, doi: 10.1080/11250009309355825.

Carrascal, L.M. & Díaz, J.A. (1989). Thermal ecology and spatio-temporal distribution of the Mediterranean lizard *Psammodromus algirus*. *Holarctic Ecology* 12, 137–143, doi: 10.1111/j.1600-0587.1989.tb00832.x.

Corti, C., Biaggini, M. & Capula, M. (2010). *Podarcis siculus* (Rafinesque-Schmaltz, 1810). In *Fauna d'Italia. Reptilia*, 407–417. Corti, C., Capula, M., Luiselli, L. & Sindaco, R. (eds). Bologna: Edizioni Calderini de Il Sole 24 Ore, Editoria Specializzata S.r.l.

Corti, C., Barbagli, F., Bassu, L., Di Cerbo, A.R., Lo Cascio, P., Sillero, N., Nulchis, V., Ceccolini, F., Paggetti, E., Romano, A., Bazzoffi, P. & Biaggini, M. (2015). Monitoraggio della biodiversità in relazione all'applicazione degli standard di condizionalità: 4.2c, 4.6, 4.3 (olivo). Technical Report. *Italian Journal of Agronomy* 10(s1), doi: 10.4081/ija.2015.749.

Díaz, J.A. & Carrascal, L.M. (1991). Regional distribution of a Mediterranean lizard: influence of habitat cues and prey abundance. *Journal of Biogeography* 18, 291–297.

Díaz, J.A., Monasterio, C. & Salvador, A. (2006). Abundance, microhabitat selection and conservation of eyed lizards (*Lacerta lepida*): a radiotelemetric study. *Journal of Zoology* 268, 295–301, doi: 10.2307/2845399.

Diego-Rasilla, F.J. (2003). Influence of predation pressure on the escape behavior of *Podarcis muralis* lizards. *Behavioural Processes* 63, 1–7, doi: 10.1016/S0376-6357(03)00026-3.

Driscoll, D.A. (2004). Extinction and outbreaks accompany

fragmentation of a reptile community. *Ecological Applications* 14, 220–240, doi: 10.1890/02-5248.

Foà, A., Bearzi, M. & Baldaccini, N.E. (1990). A preliminary report on the size of the home range and on the orientational capabilities in the lacertid lizard *Podarcis sicula*. Thirteen Meeting of the Italian Society for the Study of Animal Behaviour, Perugia, Italy, May 25–27, 1989. *Ethology Ecology & Evolution* 3(2), 310, doi: 10.1080/08927014.1990.9525443.

Foà, A., Tosini, G. & Avery, R. (1992). Seasonal and diel cycles of activity in the ruin lizard, *Podarcis sicula*. *Herpetological Journal* 2, 86–89.

Gibbons, J.W., Scott, D.E., Ryan, T.J., Buhlmann, K.A., Tuberville, T.D., Metts, B.S., Greene, J.L., Mills, T., Leiden, Y., Poppy, S. & Winne, C.T. (2000). The Global Decline of Reptiles, Déjà Vu Amphibians. *BioScience* 50(8), 653–666, doi: 10.1641/0006-3568(2000)050[0653:TGDORD]2.0.CO;2.

Huey, R.B. (1982). Temperature, physiology, and the ecology of reptiles. In, Gans, C. & Pough, F.H. (Eds). *Biology of the Reptilia*, vol. 12, pp. 25–91. Academic Press, London.

Huey, R.B. & Slatkin, M. (1976). Costs and benefits of lizard thermoregulation. *The Quarterly Review of Biology* 5, 363–384, doi: 10.1086/409470.

Kleijn, D., Rundlöf, M., Scheper, J., Smith, H.G. & Tscharntke, T. (2011). Does conservation on farmland contribute to halting the biodiversity decline. *Trends in Ecology and Evolution* 28, 474–481.

Martín, J. & López, P. (1990). Amphibians and reptiles as prey of birds in southwestern Europe. *Smithsonian Herpetological Information Service* 82, 1–43.

Martín, J. & López, P. (1996). Avian predation on a large lizard (*Lacerta lepida*) found at a low population densities in Mediterranean habitats: an analysis of bird diets. *Copeia* 1996, 722–726.

Martín, J. & López, P. (1998). Shifts in microhabitat use by the lizard *Psammodromus algirus*: responses to seasonal changes in vegetation structure. *Copeia* 1998, 780–786, doi: 10.2307/1447815.

Mizsei, E., Fejes, Z., Malatinszky, Á., Lengyel, S. & Vadász, C. (2020). Reptile responses to vegetation structure in a grassland restored for an endangered snake. *Community Ecology* 21, 203–212, doi: 10.1007/s42974-020-00019-2.

Nopper, J., Lauströer, B., Rödel, M.O. & Ganzhorn, J.U. (2017). A structurally enriched agricultural landscape maintains high reptile diversity in sub-arid south-western Madagascar. *Journal of Applied Ecology* 54, 480–488, doi: 10.1111/1365-2664.12752.

Ockleford, C., Adriaanse, P., Berny, P., Brock, T., Duquesne, S., Grilli, S., Hernandez J.A., Bennekou, S., Klein, M., Kuhl, T., Laskowski, R., Machera, K., Pelkonen, O., Pieper, S., Stemmer, M., Sundh, I., Teodorović, I., Tiktak, A., Topping, C. & Smith, R. (2018). Scientific Opinion on the state of the science on pesticide risk assessment for amphibians and reptiles. *EFSA Journal* 16(2), doi: 10.2903/j.efsa.2018.5125.

Pérez-Mellado, V. & Corti, C. (1993). Dietary adaptation and herbivory in lacertid lizards of the genus *Podarcis* from western Mediterranean islands. (Reptilia: Sauria). *Bonner Zoologische Beiträge* 44, 193–220.

Regal, P.J. (1983). The adaptive zone and behaviour of lizards. In, Huey, R.B., Pianka, E.R., Schoener, T.W. (Eds). *Lizard Ecology: Studies of a Model Organism*, pp. 105–118.



- Harvard University Press, Cambridge, MA, doi: 10.4159/harvard.9780674183384.
- Rugiero, L. (1994). Food habits of the ruin lizard, *Podarcis sicula* (Rafinesque-Schmaltz, 1810) from a coastal dune in central Italy (Squamata: Sauria: Lacertidae). *Herpetozoa* 7, 71–73, doi: 10.1111/j.1469-7998.1966.tb03886.x.
- Rugiero, L. (1997). Tail loss and escape behaviour in the Common Wall Lizard *Podarcis muralis* Laurenti, 1768. A preliminary analysis (Squamata: Sauria: Lacertidae). *Herpetozoa* 10 (3/4), 149–152.
- Sala, O.E., Chapin III, F.S., Armesto, J.J., Berlow, E., Bloomfield, J. Dirzo, R., Huber-Sanwald, E., Huenneke, L.F., Jackson, R.B., Kinzig, A., Leemans, R., Lodge, D.M., Mooney, H.A. & Wall, D.H. (2000). Global biodiversity scenarios for the year 2100: *Science* 287(5459), 1770–1774, doi: 10.1126/science.287.5459.1770.
- StatSoft, Inc. (2011). STATISTICA (data analysis software system), version 10. www.statsoft.com.
- Strijbosch, H., 1988. Habitat selection of *Lacerta vivipara* in a lowland environrnt. *Herpetological Journal* 1, 207–210.
- Tews, J., Brose, U., Grimm, V., Tielbörger, K., Wichmann, M. C., Schwager, M. & Jeltsch, F. (2004). Animal species diversity driven by habitat heterogeneity/diversity: the importance of keystone structures. *Journal of Biogeography* 31, 79–92.
- Todd, B.D., Willson, J.D. & Gibbons, J.W. (2010). The global status of reptiles and causes of their decline. In *Ecotoxicology of Amphibians and Reptiles*, Second Edition, 47–67. Sparling, D.W., Bishop, C.A. & Krest, S. (eds). Pensacoda, FL, USA: CRC Press.

- Tscharntke, T., Klein, A.M., Kruess, A., Steffan-Dewenter I. & Thies, C. (2005). Landscape perspectives on agricultural intensification and biodiversity - ecosystem service management. *Ecology Letters* 8, 857–74, doi: 10.1111/j.1461-0248.2005.00782.x.
- Van Damme, R., Bauwens, D., Castilla, A.M. & Verheyen, R. (1990). Comparative thermal ecology of the sympatric lizards *Podarcis tiliguerta* and *Podarcis sicula*. *Acta Oecologica* 11, 503–512.
- Vitt, L.J., Colli, G.R., Caldwell, J.P., Mesquita, D.O., Garda, A.A. & França, F.G.R. (2007). Detecting variation in microhabitat use in low-diversity lizard assemblages across small-scale habitat gradients. *Journal of Herpetology* 41(4), 654–663, doi: 10.1670/06-279.1.
- Wisler C., U., Hofer & Arlettaz, R.L. (2008). Snakes and Monocultures: Habitat selection and movements of female Grass Snakes (*Natrix natrix* L.) in an agricultural landscape. *Journal of Herpetology* 42(2), 337–346, doi: 10.1670/07-027.1.

Accepted: 18 December 2020

## Influence of the presence of invasive mosquitofish and submerged vegetation on oviposition site selection by gray treefrogs (*Hyla versicolor*)

Geoffrey R. Smith<sup>1</sup> & Jessica E. Rettig<sup>1</sup>

<sup>1</sup> Department of Biology, Denison University, Granville, OH 43023 USA

Amphibians often select oviposition sites based on a variety of cues that indicate the level of risk in the oviposition habitat. Surprisingly, the role of aquatic vegetation or habitat structure/complexity in anuran oviposition site selection has not been extensively studied even though it might affect perceived risk. We examined the effects of free-ranging invasive western mosquitofish (*Gambusia affinis*) and artificial vegetation/habitat structure on colonisation of experimental pools by gray treefrogs (*Hyla versicolor*). *Hyla versicolor* avoided ovipositing in mesocosms with *G. affinis*. The presence of artificial vegetation/habitat structure had no effect on oviposition site selection by *H. versicolor*, whether alone or in interaction with *G. affinis*. Our experiment provides evidence for the avoidance of fish, and more specifically *G. affinis*, by ovipositing *H. versicolor*; but provides no evidence for a role of vegetation/habitat structure.

**Keywords:** Eggs, *Gambusia affinis*, habitat complexity, *Hyla versicolor*, oviposition, vegetation

## INTRODUCTION

Adults of amphibians that breed in aquatic habitats often select oviposition sites based on a variety of cues indicating the level of risk to their offspring, including predation (Pintar & Resetarits, 2017b; Resetarits et al., 2018; Smith & Harmon, 2019), desiccation or water holding potential (Rudolf & Rödel, 2005; Pintar & Resetarits, 2017b), competition (Schulte et al., 2011; Stein & Blaustein, 2015; Smith & Harmon, 2019), conspecifics (Rudolf & Rödel, 2005), and parasitism (Kiesecker & Skelly, 2000) (see also review in Blaustein, 1999; Buxton & Sperry, 2017). For most of these cues, the parent selects an oviposition site that maximises the benefit to their offspring (Pintar & Resetarits, 2017a; Hawley Matlaga, 2018), but not always perhaps due to shifts in the offspring environment indicated by the cue (e.g., historically, low levels of tannins indicated a temporary pond without fish but more recently high tannin levels in pond due to invasive plants are harmful to offspring; Dodd & Buchholz, 2018).

Surprisingly, the effect of habitat structure and complexity, including the presence of submerged and surrounding vegetation, on oviposition site selection has rarely been experimentally studied to our knowledge. However, there have been some observational studies and field experiments that have examined this and related questions. Natural oviposition sites (i.e., pools, wetlands, ditches) of the rice frog (*Fejervarya*

*limnocharis*) had slightly higher vegetation cover (4 %) than sites not used for oviposition (Xu & Li, 2013). Oregon spotted frogs (*Rana pretiosa*) appear to prefer to oviposit in flooded wetlands that have had reed canary grass (*Phalaris arundinacea*) removed by mowing compared to control wetlands (Kapust et al., 2012). In a field study, ponds in which adult green and golden bell frogs (*Litoria aurea*) bred had more aquatic vegetation on average than ponds in which they did not breed (Klop-Toker et al., 2016). Glos et al. (2008) found that oviposition by *Aglyptodactylus laticeps* in artificial ponds was not affected by the presence or absence of leaf litter, but vegetative structure was not directly manipulated.

On its own, aquatic vegetation might be expected to affect the suitability of a habitat for tadpoles. Females may choose sites with more aquatic vegetation as it can have a positive effect on offspring success. For example, aquatic vegetation may increase the feeding rate and food consumption of tadpoles by affecting the profitability of the habitat, at least during some times of the day (Warkentin, 1992). In addition, the effects of a predator on oviposition might be mediated by the presence of vegetation or habitat structure. For example, the presence of aquatic vegetation can increase the survivorship of tadpoles in the presence of various invertebrate predators, possibly due to reducing the ability of predators to detect prey or by reducing capture success of the predator (e.g., Babbitt & Tanner, 1997, 1998; Tarr & Babbitt, 2002; Kopp et al., 2006; Cuervo

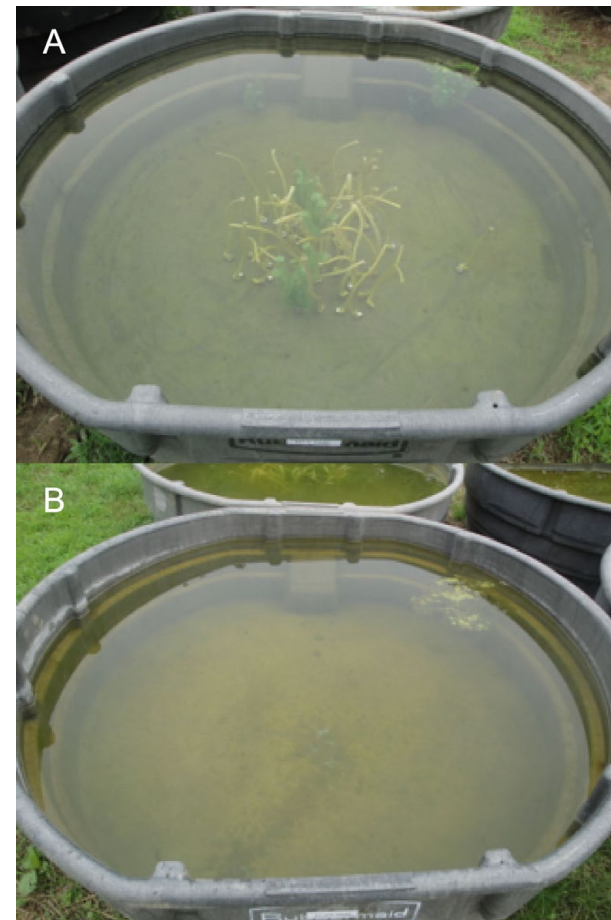
et al., 2017). However, the efficiency of some tadpole predators is unaffected by vegetation cover, perhaps by providing the predator with a means of reaching their prey (e.g., crayfish, Figiel & Semlitsch, 1991; Chandler et al., 2016). Thus, the presence of vegetation, depending on how it might affect predation risk for tadpoles, may or may not affect oviposition site selection by adult anurans. The western mosquitofish (*Gambusia affinis*) is a widely introduced, invasive fish that has had significant negative effects on amphibian populations, primarily as a predator on their early aquatic life stages (review in Pyke, 2008). Hylids often avoid ovipositing in experimental ponds with *G. affinis*, presumably through detection of chemical or physical cues (e.g., Cope's gray treefrog, *H. chrysoscelis*, Binckley & Resetarits, 2003; gray treefrog, *H. versicolor*, Smith & Harmon, 2019, *Litoria aurea*, Pollard et al., 2017; western chorus frog, *Pseudacris triseriata*, Buxton et al., 2017). In addition, the presence of *G. affinis* in constructed wetlands negatively affected the abundance of *H. versicolor* (Shulse et al., 2013). This avoidance of ponds with *G. affinis* may be due to female frogs avoiding ovipositing in risky habitats since *G. affinis* are known to prey upon and greatly reduce the abundance of hylid tadpoles (Fryxell et al., 2015; Smith & Smith, 2015; Smith & Harmon, 2019). However, not all species of frogs appear to avoid ovipositing in habitats with mosquitofish. For example, Klop-Toker et al. (2018) found adult *Litoria aurea* at ponds with and without *Gambusia holbrooki*, but tadpoles were only found in ponds without *G. holbrooki*, suggesting that these adult frogs likely breed in both types of ponds (see also Klop-Toker et al., 2016).

The potential for aquatic vegetation to mediate the effect of *G. affinis* on oviposition site selection of anurans is not clear. Some studies found aquatic vegetation or habitat complexity reduced the ability of *Gambusia* to prey upon tadpoles (Morgan & Buttemer, 1996; Baber & Babbitt, 2004), even to the point of allowing coexistence (Preston et al., 2017). Thus, for adult anurans, the presence of aquatic vegetation might be a cue to lower predation risk for their offspring, and thus might affect their oviposition site selection. However, other studies found the presence of aquatic vegetation or habitat complexity had no effect on the injury rate (Shulse & Semlitsch, 2014) or consumption (Baber & Babbitt, 2004) of tadpoles by *Gambusia*. Indeed, *Gambusia* can be found in open water and in areas with submerged vegetation (Casterlin & Reynolds, 1977; Klop-Toker et al., 2018; review in Pyke, 2008), and *G. affinis* is found in both open water and vegetated habitats in local ponds (G.R. Smith & J.E. Rettig, unpubl. data), and are effective predators of tadpoles in both open or vegetated habitats, even if the vegetation does provide some refuge for tadpoles (Morgan & Buttemer, 1996; Baber & Babbitt, 2004). Thus, even though aquatic vegetation may provide some protection for tadpoles from predation by *Gambusia*, the effect of such predation is still potentially high enough to cause reduced abundances of tadpoles even in ponds with aquatic vegetation. Thus, it appears that the effects of aquatic vegetation on oviposition site selection by anurans may or may not be related to the presence of *G. affinis*.

We examined the effects of the presence of *G. affinis* and artificial vegetation on the oviposition site selection of free-ranging *H. versicolor*. *Hyla versicolor* is typically found in open marshes and in ponds with emergent vegetation (Collins & Wilbur, 1979). Vegetation cover positively affected *H. versicolor* tadpole abundance (Shulse et al., 2013), and appeared to reduce extinction probabilities of *H. versicolor/H. chrysoscelis* in wetlands, and may be important for egg deposition or as calling sites (Grant et al., 2018). However, the presence of aquatic vegetation in constructed wetlands had no effect on the abundance of *H. versicolor* in the presence of *G. affinis* (Shulse et al., 2013). *Hyla versicolor/H. chrysoscelis* may also be found in a range of pond hydroperiods from temporary to permanent ponds (Collins & Wilbur, 1979; Kiesecker & Skelly, 2001; Pauley, 2011). Mosquitofish readily colonise a variety of ponds and wetlands, including temporary ponds (Alemadi & Jenkins, 2008; see also Pyke, 2005). Indeed, we have observed *G. affinis* repeatedly colonise a local temporary pond after spring flooding, including ponds used by *H. versicolor* (G.R. Smith & J.E. Rettig, pers. observ.). Thus, *G. affinis* and *H. versicolor* potentially encounter each other in a variety of ponds where their distributions overlap. We predicted *H. versicolor* would avoid ovipositing in mesocosms with *G. affinis*. We also predicted greater oviposition by *H. versicolor* in mesocosms with vegetation. Given the apparent effects of vegetation on *G. affinis* predation on tadpoles in previous studies (see above), we predicted that the presence of vegetation would not affect avoidance of mesocosms with *G. affinis*.

## MATERIALS AND METHODS

We used 32 Rubbermaid cattletanks (1136 L capacity; height = 63.5 cm, width = 175 cm, length = 160 cm) as experimental ponds. Our experiment consisted of a 2 x 2 factorial experiment in which the presence/absence of *G. affinis* and artificial vegetation were included as factors. Each treatment combination was replicated 8 times. We placed mesocosms in eight blocks of four mesocosms so that each treatment combination was present in each block. All blocks were placed at least 5 m apart within the same fenced area on the Denison University Biological Reserve, Granville, Licking County, Ohio. All mesocosms were filled with 800 L of well water to a depth of 44 cm (within the depth of local ponds used by these species) on 6 and 7 July 2013 and covered with 1 mm window screening to prevent colonisation by invertebrates and amphibians. On 8, 12, and 15 July, we inoculated all tanks with water (strained through 1 mm window screening) from nearby, natural ponds, and on 9 July 2013 we added 30 g of Rabbit Chow (Purina, St. Louis, MO) to introduce zooplankton and algae to the mesocosms and to facilitate algal growth. To ponds assigned to having vegetation present, we added submerged artificial vegetation in the form of lengths of nylon rope (40 cm long, 1 cm wide) and plastic aquarium plants (variety of "species"; Tetra, Blacksburg, Virginia, USA) weighed down with stainless steel nuts at the center of the mesocosm which provided a relatively dense vegetated area of rope and artificial



**Figure 1.** Photographs of representative mesocosms A) with artificial vegetation and B) without artificial vegetation..

plants through most of the water column (Fig. 1). The artificial aquatic vegetation "species" were chosen because they were similar in appearance and general structure to natural vegetation we have observed in local ponds, thus attempting to make the habitat more realistic. We used nylon rope to provide additional structure and density to supplement the artificial vegetation. To ponds assigned to having no vegetation present, we did not add either rope or artificial vegetation. We added 5 female *G. affinis* (45-55 mm total length) to each appropriate mesocosm on 18 July 2013 and removed the window screening to begin the experiment. The number of *G. affinis* added to the mesocosms is within the observed densities of *G. affinis* in local ponds (J.E. Rettig and G.R. Smith, unpubl. data).

Once the experiment began, we carefully searched each mesocosm for egg masses every morning shortly after sunrise from 19 July to 28 July. Our visual searching encompassed the entire surface of the water in each mesocosm, as well as the entire water column, the sides of the mesocosm, and the artificial vegetation (if present). We visually counted eggs using a hand-held counter. Female *H. versicolor* lay multiple clusters of eggs ranging in size from 30 to 40 (Cline, 2005), making counting eggs by eye easier than for other types of egg masses. We did not remove eggs from the mesocosms after counting. We were able to differentiate newly laid eggs from older eggs based on changes in the appearance of eggs and

their jelly coat, and only counted freshly laid eggs each morning. Embryo development (i.e., elongation) typically became obvious within 48-72 h after oviposition. Since mosquitofish are visual foragers (Russo et al., 2008 and references therein), and show a morning peak in foraging (Pyke, 2005), the opportunity for them to consume eggs before our morning egg surveys was limited. We never observed the fish consuming eggs during our daily checks. In addition, *G. affinis* do not consume *H. versicolor* eggs in our study population, only hatchlings and tadpoles (Smith & Smith, 2015). Therefore the egg counts we made are unlikely to have been affected by consumption of eggs by the fish. Allowing the eggs to remain in the mesocosms after counting could have affected the oviposition decisions of adult *H. versicolor* if they avoid potential competitors for their offspring (see Rudolf & Rödel, 2005; Schulte et al., 2011; Stein & Blaustein, 2015; Smith & Harmon, 2019). However, this is unlikely to have been very influential in our short experiment. In particular, the mesocosms did not appear to be saturated by eggs (e.g., some fishless mesocosms had not received eggs by the end of the experiment; see Results), and there were two cases when eggs were laid in mesocosms that already had eggs deposited earlier in the experiment. For mesocosms receiving eggs, we calculated the mean day of the experiment when eggs were deposited.

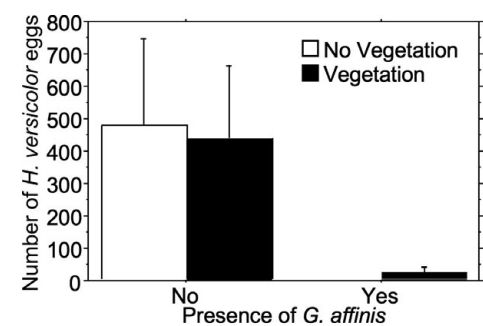
We used a two-way ANOVA to examine the effects of fish and vegetation treatments on the number of eggs laid in a mesocosm ( $\log[x+1]$  transformed to meet the assumptions of parametric analyses). In a preliminary analysis the effect of pond block was not significant and thus has not been presented. We used JMP Pro 14.1 for all data analyses. Means are given  $\pm 1$  S.E.

## RESULTS

We observed a total of 7,528 eggs in all mesocosms. We counted a total of 7,360 eggs in mesocosms with no *G. affinis* ( $n = 16$  mesocosms) and 168 eggs in mesocosms with *G. affinis* ( $n = 16$  mesocosms). A total of 3,850 eggs were deposited in mesocosm without artificial vegetation ( $n = 16$  mesocosms) and 3,678 eggs were deposited in mesocosms with artificial vegetation ( $n = 16$  mesocosms). We observed a total of 3,850 eggs in mesocosms with no *G. affinis* and no artificial vegetation ( $n = 8$  mesocosms), 3510 eggs in mesocosms with no *G. affinis* and artificial vegetation ( $n = 8$  mesocosms), zero eggs in mesocosms with *G. affinis* and no artificial vegetation ( $n = 8$  mesocosms), and 168 eggs in mesocosms with *G. affinis* and aquatic vegetation ( $n = 8$  mesocosms). We observed oviposition in three mesocosms with no *G. affinis* and no artificial vegetation, three mesocosms with no *G. affinis* and with artificial vegetation, no mesocosms with *G. affinis* and no artificial vegetation, and one mesocosm with *G. affinis* and with artificial vegetation.

There were significantly fewer *H. versicolor* eggs deposited in mesocosms with mosquitofish than were deposited in mesocosms without mosquitofish (Fig. 2;  $F_{1,28} = 5.74$ ,  $P = 0.024$ ). The presence of artificial vegetation had no effect on the number of *H. versicolor*





**Figure 2.** The effect of the interaction of the western mosquitofish (*Gambusia affinis*) and artificial vegetation treatments on mean ( $\pm 1$  S.E.) number of gray treefrog (*Hyla versicolor*) eggs laid in mesocosms.

eggs deposited in a mesocosm (Fig. 2;  $F_{1,28} = 0.11$ ,  $P = 0.74$ ). There was no significant interaction between the mosquitofish and vegetation treatments (Fig. 2;  $F_{1,28} = 0.11$ ,  $P = 0.74$ ).

When considering only mesocosms in which oviposition occurred, eggs were laid on average on day  $4.4 \pm 0.8$  of the experiment in mesocosms without *G. affinis* ( $n = 6$ ) and on day 5 in mesocosms with *G. affinis* ( $n = 1$ ). Eggs were laid on average on day  $3.8 \pm 0.6$  in mesocosms without artificial vegetation ( $n = 3$ ) and day  $5.0 \pm 1.1$  in mesocosms with artificial vegetation ( $N = 4$ ).

## DISCUSSION

Our experiment demonstrates that *H. versicolor* avoid ovipositing in mesocosms with free-ranging *G. affinis*. The reduction in oviposition by *H. versicolor* in mesocosms with free-ranging *G. affinis* is consistent with other studies on anurans. *Hyla versicolor* and *H. chrysoscelis* avoid ovipositing in ponds with fish in general (Vonesh et al., 2009; Kraus & Vonesh, 2010; Kraus et al., 2011), and *G. affinis* and *G. holbrooki* specifically (Binckley & Resetarits, 2003; Smith & Harmon, 2019). The mechanism by which *H. versicolor* avoid *G. affinis* is unknown (i.e., is it due to detection of chemical or physical cues?). However, in another experiment, *H. versicolor* laid fewer eggs in the mesocosms with free-ranging *G. affinis* compared to mesocosms with caged *G. affinis* and laid eggs in mesocosms with caged *G. affinis* later than in mesocosms with no *G. affinis* (Smith & Harmon, 2019), suggesting both chemical and physical cues and likely resulted in the avoidance of *G. affinis* by ovipositing adults. The avoidance of *G. affinis* is likely adaptive since tadpoles of *H. versicolor* are readily consumed by *G. affinis* (Grubb, 1972; Smith & Smith, 2015) and few tadpoles persist in the presence of *G. affinis* (Shulze et al., 2013; Smith & Harmon, 2019). Thus, avoidance of ponds with free-ranging *G. affinis* is an example of risk-sensitive oviposition (Buxton & Sperry, 2017).

As far as we know, our study is the first to experimentally examine the effect of vegetation or habitat structure on oviposition site selection in *H. versicolor*. We found no evidence for its role in oviposition site selection, nor for a role for the interaction of

vegetation and predator presence. The lack of any effect of artificial vegetation/habitat structure on oviposition by *H. versicolor* in our experiment may arise because either the *H. versicolor* did not perceive the presence of the vegetation/habitat structure (i.e., not sufficiently realistic or not sufficiently abundant) or they did not perceive a benefit (or cost) from the vegetation/habitat structure, even though they are often found in ponds with aquatic vegetation in nature (e.g., Shulze et al., 2013; Grant et al., 2018). For example, vegetation structure had no effect on survival of larval *H. versicolor* in a mesocosm experiment in the absence of predators (Purrenhage & Boone, 2009), suggesting that aquatic vegetation on its own does not provide a benefit for tadpole survivorship in *H. versicolor*. In addition, the presence of aquatic vegetation in constructed wetlands does not appear to influence the abundance or presence of *H. versicolor/chrysoscelis* complex (Shulze et al., 2010). Thus, the presence of vegetation may not actually be a cue for a beneficial environment, at least relative to other cues.

We found no significant interaction between the presence of *G. affinis* and artificial vegetation on the number of eggs laid in mesocosms by *H. versicolor*, suggesting that the presence of the artificial vegetation did not mediate the effect of *G. affinis* on oviposition site selection by *H. versicolor*. However, *H. versicolor* did lay some eggs (total = 168 eggs) in mesocosms with both *G. affinis* and artificial vegetation, and none in mesocosms with *G. affinis* and no artificial vegetation. Thus, there may be a slight effect on oviposition site selection by *H. versicolor* but this is very minor relative to the contrast between the presence and absence of *G. affinis*. In addition, *G. affinis* tends to use submerged vegetation (Casterlin & Reynolds, 1977), and thus vegetation may also not provide any protection from *G. affinis*. For example, structural complexity did not affect or had minimal effects on predation rates on tadpoles (e.g., *Litoria aurea*, bleating tree frog, *Litoria dentata*, Morgan & Buttemer, 1996; squirrel treefrog, *Hyla squirella*, Baber & Babbitt, 2004) or lower the injury rate of tadpoles (*Rana* spp., Shulze & Semlitsch, 2014) in the presence of *G. affinis*. In addition, the presence of aquatic vegetation in constructed wetlands had no effect on the abundance of *H. versicolor* in the presence of *G. affinis* (Shulze et al., 2013). However, the presence of aquatic macrophytes reduced predation of *G. affinis* on larval northern Pacific treefrog (*Pseudacris regilla*) in mesocosms which might explain how *P. regilla* coexisted with *G. affinis* in a lake experiment (Preston et al., 2017). Thus, 1) the presence of vegetation may not change the perception of predation risk due to *G. affinis* in a habitat or 2) the effects of the presence of vegetation on perceived predation risk by *G. affinis* may be variable and unpredictable, and thus may not be a useful cue for oviposition site choice in *H. versicolor*.

In conclusion, our experiment confirms the avoidance of fish, and more specifically *G. affinis*, by ovipositing *H. versicolor*. However, our experiment found no evidence for a role of vegetation/habitat structure in oviposition site selection in *H. versicolor*, whether on its own or mediating the effect of *G. affinis*, which suggests that

the presence of aquatic vegetation in ponds may not have benefits for the success of offspring. Our results therefore emphasise the necessity of maintaining wetlands, including temporary wetlands that *G. affinis* can colonise, free of non-native fish such as *G. affinis* to preserve viable populations of *H. versicolor*. Our results also suggest that submergent vegetation may not be used for initial oviposition site selection by *H. versicolor* and may not help mediate the negative effects of *G. affinis* on oviposition site selection. We recommend additional experiments on other species of amphibians, as well as experiments considering a range of fish densities and vegetation/habitat structure, especially experiments that use natural vegetation or that vary vegetation density or habitat complexity, to confirm or refine our results and conclusions.

## ACKNOWLEDGEMENTS

This research was conducted under permit from the Ohio Department of Wildlife (14-128) and was approved by the Denison University Institutional Animal Care and Use Committee (09-007). We thank W. and L. Smith for help during the experiment and V. Buxton, J. Valdez, and an anonymous reviewer for helpful comments on this manuscript.

## REFERENCES

Alemadi, S.D. & Jenkins, D.G. (2008). Behavioral constraints for the spread of the eastern mosquitofish, *Gambusia holbrooki* (Poeciliidae). *Biological Invasions* 10, 59-66.

Baber, M.J. & Babbitt, K.J. (2004). Influence of habitat complexity on predator-prey interactions between the fish (*Gambusia holbrooki*) and tadpoles of *Hyla squirella* and *Gastrophryne carolinensis*. *Copeia* 2004, 173-177.

Babbitt, K.J. & Tanner, G.W. (1997). Effects of cover and predator identity on predation of *Hyla squirella* tadpoles. *Journal of Herpetology* 31, 128-130.

Babbitt, K.J. & Tanner, G.W. (1998). Effects of cover and predator size on survival and development of *Rana utricularia* tadpoles. *Oecologia* 114, 258-262.

Binckley, C.A. & Resetarits, W.J. Jr. (2003). Functional equivalence of non-lethal effects: Generalized fish avoidance determines distribution of gray treefrog, *Hyla chrysoscelis*, larvae. *Oikos* 102, 623-629.

Blaustein, L. (1999). Oviposition site selection in response to risk of predation: Evidence from aquatic habitats and consequences for population dynamics and community structure. In Wasser, S.P. (Ed.), *Evolutionary Theory and Processes: Modern Perspectives, Papers in Honour of Eviatar Nevo* (pp. 441-456). The Netherlands: Kluwer Academic Publishers.

Buxton, V.L. & Sperry, J.H. (2017). Reproductive decision in anurans: A review of how predation and competition affects the deposition of eggs and tadpoles. *BioScience* 67, 25-37.

Buxton, V.L., Ward, M.P. & Sperry, J.H. (2017). Frog breeding pond selection in response to predators and conspecific cues. *Ethology* 123, 397-404.

Casterlin, M.E. & Reynolds, W.W. (1977). Aspects of habitat selection in the mosquitofish *Gambusia affinis*.

*Hydrobiologia* 55, 125-127.

Chandler, H.C., Gorman, T.A. & Haas, C.A. (2016). The effects of crayfish predation and vegetation cover on tadpole growth, survival, and nonlethal injury. *Journal of Herpetology* 50, 271-277.

Cline, G.R. (2005). *Hyla versicolor* La Conte, 1825. Eastern Gray Treefrog. In Lannoo, M. (Ed.), *Amphibian Declines: The Conservation Status of United States Species* (pp. 458-461). California: University of California Press.

Collins, J.P. & Wilbur, H.M. (1979). Breeding habits and habitats of the amphibians of the Edwin S. George Reserve, Michigan, with notes on the local distribution of fishes. *Occasional Papers of the Museum of Zoology, University of Michigan* 686, 1-34.

Cuello, M.E., Úbeda, C.A. & Bello, M.T. (2017). Habitat associations for the endangered frog *Atelognathus patagonicus* within the aquatic environment: Key microhabitats for conservation. *Herpetological Conservation and Biology* 12, 410-421.

Dodd, C.E. & Buchholz, R. (2018). Apparent maladaptive oviposition site choice of Cope's Gray Treefrogs (*Hyla chrysoscelis*) when offered an array of pond conditions. *Copeia* 106, 492-500.

Figiel, C.R. Jr. & Semlitsch, R.D. (1991). Effects of nonlethal injury and habitat complexity on predation in tadpole populations. *Canadian Journal of Zoology* 69, 830-834.

Fryxell, D.C., Arnett, H.A., Apgar, T.M., Kinnison, M.T. & Palkovacs, E.P. (2015). Sex-ratio variation shapes the ecological effects of a globally introduced freshwater fish. *Proceedings of the Royal Society* 282B, 20151970.

Glos, J., Wagner, F., Dausmann, K.H. & Linsenmair, K.E. (2008). Oviposition-site selection in an endangered Madagascan frog: Experimental evaluation of a habitat model and its implications for conservation. *Biotropica* 40, 646-652.

Grant, T.J., Otis, D.L. & Koford, R.R. (2018). Comparison between anuran call only and multiple life-stage occupancy designs in Missouri River floodplain wetlands following a catastrophic flood. *Journal of Herpetology* 52, 371-380.

Grubb, J.C. (1972). Differential predation by *Gambusia affinis* on the eggs of seven species of anuran amphibians. *American Midland Naturalist* 88, 102-108.

Hawley Matlaga, T.J. (2018). Mechanisms underlying the occurrence of species in complex modified tropical landscapes: a case study of amphibians in the Osa Peninsula, Costa Rica. *Journal of Tropical Ecology* 34, 32-40.

Kapust, H.Q.W., McAllister, K.R. & Hayes, M.P. (2012). Oregon Spotted Frog (*Rana pretiosa*) response to enhancement of oviposition habitat degraded by invasive reed canary grass (*Phalaris arundinacea*). *Herpetological Conservation and Biology* 7, 358-366.

Kiesecker, J.M. & Skelly, D.K. (2000). Choice of oviposition site by Gray Treefrogs: The role of potential parasitic infection. *Ecology* 81, 2939-2943.

Kiesecker, J.M. & Skelly, D.K. (2001). Effects of disease and pond drying on gray treefrog growth, development, and survival. *Ecology* 82, 1956-1963.

Klop-Toker, K., Valdez, J., Stockwell, M., Clulow, S., Clulow, J., & Mahony, M. (2018). Community level impacts of invasive mosquitofish may exacerbate the impact to a threatened amphibian. *Austral Ecology* 43, 213-224.

Klop-Toker, K., Valdez, J., Stockwell, M., Fardell, L., Clulow, S.,

- Clulow, J. & Mahony, M. (2016). We made your bed, why won't you lie in it? Food availability and disease may affect reproductive output of reintroduced frogs. *Plos One* 11(7), e0159143.
- Kopp, K., Wachievski, M., & Eterovick, P.C. (2006). Environmental complexity reduces tadpole predation by water bugs. *Canadian Journal of Zoology* 84, 136-140.
- Kraus, J.M., & Vonesh, J.R. (2010). Feedbacks between community assembly and habitat selection shape variation in local colonization. *Journal of Animal Ecology* 79, 795-802.
- Kraus, J.M., Pletcher, L.T., & Vonesh, J.R. (2011). Variation in active and passive resource inputs to experimental pools: Mechanisms and possible consequences for food webs. *Freshwater Biology* 56, 491-502.
- Morgan, L.A., & Buttemer, W.A. (1996). Predation by the non-native fish *Gambusia holbrooki* on small *Litorea aurea* and *L. dentata* tadpoles. *Australian Zoologist* 30, 143-149.
- Pauley, T.K. (2015). Amphibians in the Canaan Valley Drainage. *Southeastern Naturalist* 14, 314-322.
- Pintar, M.R. & Resetarits, W.J. Jr. (2017a). Out with the old, in with the new: Oviposition preference matches larval success in Cope's Gray Treefrog, *Hyla chrysoscelis*. *Journal of Herpetology* 51, 186-189.
- Pintar, M.R. & Resetarits, W.J. Jr. (2017b). Relative predation risk and risk of desiccation co-determine oviposition preferences in Cope's Gray Treefrog, *Hyla chrysoscelis*. *Oecologia* 184, 423-430.
- Pollard, C.J., Stockwell, M.P., Bower, D.S., Garnham, J.I., Pickett, E.J., Darcovich, K., O'Meara, J., Clulow, J. & Mahony, M.J. (2017). Removal of an exotic fish influences amphibian breeding site selection. *Journal of Wildlife Management* 81, 720-727.
- Preston, D.L., Hedman, H.D., Esfahani, E.R., Pena, E.M., Boland, C.E., Lunde, K.B. & Johnson, P.T.J. (2017). Responses of a wetland ecosystem to the controlled introduction of invasive fish. *Freshwater Biology* 62, 767-778.
- Purrenhage, J.L. & Boone, M.D. (2009). Amphibian community response to variation in habitat structure and competitor density. *Herpetologica* 65, 14-30.
- Pyke, G.H. (2005). A review of the biology of *Gambusia affinis* and *G. holbrooki*. *Reviews in Fish Biology and Fisheries* 15, 339-365.
- Pyke, G.H. (2008). Plague minnow or mosquito fish? A review of the biology and impacts of introduced *Gambusia* species. *Annual Review of Ecology, Evolution and Systematics* 39, 171-191.
- Resetarits, W.J. Jr., Bohenek, J.R., Breech, T. & Pintar, M.R. (2018). Predation risk and patch size jointly determine perceived patch quality in ovipositing treefrogs, *Hyla chrysoscelis*. *Ecology* 99, 661-669.

- Rudolf, V.H.W. & Rödel, M.-O. (2005). Oviposition site selection in a complex and variable environment: The role of habitat quality and conspecific use. *Oecologia* 142, 316-325.
- Russo, G., Chou, A., Rettig, J.E. & Smith, G.R. (2008). Foraging responses of mosquitofish (*Gambusia affinis*) to items of difference sizes and colors. *Journal of Freshwater Ecology* 23, 677-678.
- Schulte, L.M., Yeager, J., Schulte, R., Veith, M., Werner, P., Beck, L.A. & Lötters, S. (2011). The smell of success: Choice of larval rearing sites by means of chemical cues in a Peruvian poison frog. *Animal Behaviour* 81, 1147-1154.
- Shulse, C.D. & Semlitsch, R.D. (2014). Western mosquitofish (*Gambusia affinis*) bolster the prevalence and severity of tadpole tail injuries in experimental wetlands. *Hydrobiologia* 723, 131-144.
- Shulse, C.D., Semlitsch, R.D., & Trauth, K.M. (2013). Mosquitofish dominate amphibian and invertebrate community development in experimental wetlands. *Journal of Applied Ecology* 50, 1244-1256.
- Shulse, C.D., Semlitsch, R.D., Trauth, K.M. & Williams, A.D. (2010). Influences of design and landscape placement parameters on amphibian abundance in constructed wetlands. *Wetlands* 30, 915-928.
- Smith, G.R., & Harmon, J.J. (2019). Differential oviposition and offspring success of gray treefrogs in the presence of an invasive fish. *Ecosphere*, 10(2), e02612.
- Smith, G.R. & Smith, L.E. (2015). Effects of Western Mosquitofish (*Gambusia affinis*) on tadpole production of Gray Treefrogs (*Hyla versicolor*). *Herpetological Conservation and Biology* 10, 723-727.
- Stein, M. & Blaustein, L. (2015). Larval performance and oviposition habitat selection of the tree frog, *Hyla savignyi* in response to conspecific larval density. *Israel Journal of Ecology and Evolution* 61, 61-66.
- Tarr, T.L. & Babbitt, K.J. (2002). Effects of habitat complexity and predator identity on predation of *Rana clamitans* larvae. *Amphibia-Reptilia* 23, 13-20.
- Vonesh, J.R., Kraus, J.M., Rosenberg, J.S. & Chase, J.M. (2009). Predator effects on aquatic community assembly: Disentangling the roles of habitat selection and post-colonization processes. *Oikos* 118, 1219-1229.
- Warkentin, K.M. (1992). Microhabitat use and feeding rate variation in green frog tadpoles (*Rana clamitans*). *Copeia* 1992, 731-740.
- Xu, F. & Li, Y. (2013). Oviposition site selection by rice frogs on Taohua Island and the nearby mainland. *Herpetological Journal* 23, 51-53.

Accepted: 30 December 2020



<https://doi.org/10.33256/31.2.9198>

## Hidden in plain sight: detectability and habitat selection of the central plateau dusky rattlesnake in anthropized landscapes

Leroy Soria-Díaz<sup>1</sup>, Claudia C. Astudillo-Sánchez<sup>2</sup>, Yuriana Gómez-Ortiz<sup>3</sup>, Javier Manjarrez<sup>4</sup>, Víctor Mundo-Hernández<sup>4</sup>, Tamara Rubio-Blanco<sup>3</sup> & Hublester Domínguez-Vega<sup>3</sup>

<sup>1</sup> Instituto de Ecología Aplicada, Universidad Autónoma de Tamaulipas, Mexico

<sup>2</sup> Facultad de Ingeniería y Ciencias, Centro Universitario Victoria, Universidad Autónoma de Tamaulipas, Mexico

<sup>3</sup> División de Desarrollo Sustentable, Universidad Intercultural del Estado de México, Mexico

<sup>4</sup> Facultad de Ciencias, Universidad Autónoma del Estado de México, Mexico

Human activities have caused several changes in biotic communities all over the world. Some species maintain viable populations in altered environments through different adaptations, however, knowledge in this regard is scarce for certain taxa, including reptiles. We analysed the detectability and habitat selection of the rattlesnake *Crotalus triseriatus* to contribute to our knowledge about how this species responds to anthropogenic landscape change. Rattlesnakes were monitored for two years in two anthropized landscapes using visual encounter surveys. We analysed detectability in relation to climatic factors and human activity, and habitat selection was analysed in native and non-native vegetation. Our study shows that detectability of *C. triseriatus* is differentially affected in anthropized landscapes; human activity may be determinant in landscapes such as agricultural fields, whilst climatic factors may be determinant in landscapes where people are less active such as the protected areas within urban parks. The analyses of habitat selection shows that native vegetation is crucial for the persistence of *C. triseriatus* in the studied landscapes. Several strategies may allow rattlesnakes to persist in different anthropized landscapes; nonetheless, native habitat remnants should be protected in anthropized areas to conserve wildlife.

**Keywords:** Anthropized landscapes, behavioural adaptations, Mexico, native habitat remnants, urban herpetology

## INTRODUCTION

In urban areas, human activities have changed the structure and composition of biotic communities and natural ecosystem processes (McDonnell & Hahs, 2015). Some species maintain viable populations in these areas due to their adaptive capacity (McDonnell & Hahs, 2015; Pattishall & Cundall, 2009; Wong & Candolin, 2015). The effect of anthropization on wildlife behaviour has been documented mainly in mammals and birds (Gloor et al., 2001; Marzluff & Ewing, 2001; Prange et al., 2003), whilst relatively few studies have focused on reptiles (Mitchell et al., 2008). Knowledge is particularly scarce in snakes due to their relatively low density and secretive behaviour (Sullivan et al., 2017).

Behavioural adaptations stand out among the different strategies (i.e. evolved responses and proximate responses) that favour the persistence of reptiles in modified environments (e.g. activity pattern, habitat selection, and diet; López-Alcaide & Macip-Ríos, 2011). Such strategies have been directly related to survival and reproduction (Beaupre, 1995; Manjarrez, 2017; Torello-Viera et al., 2012). Otherwise, some abiotic factors such as temperatures and rainfall play a key role in snake

detectability, activity patterns and habitat selection (Lillywhite, 1987; Moreno-Rueda & Pleguezuelos, 2007). For example, in temperate zones, snakes overwinter when temperature decrease (Gregory, 1982), and in many ecosystems, an increase in rainfall is related to higher seasonal detectability due to the increased availability of prey (Torello-Viera et al., 2012). Moreover, some snakes show high plasticity in the habitat use that could be determined by factors such as the intensity of human activities, the availability of prey, presence of optimal thermoregulation sites (Huey et al., 1989; Madsen & Shine, 1996), and/or adequate shelter (Brown et al., 1982).

The western dusky rattlesnake, *Crotalus triseriatus*, is endemic to Central Mexico along the Volcanic Belt (Bryson et al., 2014; Flores-Villela & Hernández-García, 1989). Its elevational distribution is from 2,500 to 4,572 m above mean sea level (m/a.s.l.) (Campbell & Lamar, 2004; Fernández-Badillo et al., 2011; Heimes, 2016). This species has a daytime activity pattern and is mainly found in pine-oak forests, grasslands, and agricultural areas such as cattle pasture (Canseco-Márquez & Mendoza-Quijano, 2007; Fernández-Badillo et al., 2011). It is a common predator of temperate environments in central

*Correspondence:* Hublester Dominguez (hublester.dvega@gmail.com)



Mexico where it may be considered a top predator in anthropogenic areas. Like other snakes, it is affected by aversive hunting, native habitat loss, fragmentation, and climate change (Campbell & Lamar, 2004; Filippi & Luiselli, 2000; Gentili, 2004; Plummer, 2002).

Ecological studies of *C. triseriatus* are scarce (Domínguez-Guerrero et al., 2016; Güizado-Rodríguez et al., 2016; Mociño-Deloya et al., 2014), but it has been suggested that the vulnerability of *C. triseriatus* populations when faced with fragmentation and habitat urbanisation may result in a decrease of gene flow and increased extinction risk (Sunny et al., 2015). It is, therefore, necessary to collect information on the behaviour and habitat preference of *C. triseriatus* to inform conservation efforts. The objective of this study was to analyse the detectability and habitat selection of *C. triseriatus* to identify the factors that allow its occurrence in anthropized areas of central Mexico.

## METHODS

### Study area

This study was conducted in the Toluca Valley, a highly anthropized area considered the fifth largest metropolis in Mexico, with 2.3 million inhabitants (COESPO, 2020). The region has a humid temperate climate with an annual rainfall of 500 – 600 mm, 85 % is concentrated in the summer months (June to September). Average annual temperature ranges between 12 and 15 °C (García, 2004; INEGI, 2011). Toluca has experienced rapid population growth with the consequent settlement of unplanned housing and increased demand for agricultural areas. Human activities have drastically changed the distribution of native vegetation cover, generating several landscapes around the urban core. As in many cities, the most common anthropized landscapes in the urban area of Toluca include relicts of native vegetation, agricultural areas, reforested areas with exotic vegetation, and urban infrastructure.

Our study was conducted at two different landscapes in the urban area of Toluca separated by a straight-line distance of 10.5 km. We selected the study sites considering that both sites presented previous records of the rattlesnake, both include elements of the native habitat of *Crotalus triseriatus* and the non-native elements of the most common landscapes around the Urban area of Toluca (i.e. agricultural areas, reforested areas with exotic vegetation). Anthropization at these sites has been determined by different activities, thus leading to landscapes with noticeable differences in structure, composition, and management.

Site one, “El Cerrillo” (CE), is an academic area managed by the Autonomous University of the State of Mexico. This area covers approximately 123 hectares and is located 5.1 km north of the urban area of Toluca City (19°24'27" N, 99°41'40" W). This site may be considered a typical agricultural landscape (i.e. most of the area is covered by crops) and has been under this use since 1975 (45 years). CE also includes two artificial water bodies, one of them is a small protected

area of flora and fauna. The native vegetation consists of grassland (*Muhlenbergia* sp. and *Festuca* sp.) and aquatic vegetation (*Scirpus* sp. and *Typha latifolia*). The non-native vegetation consists of crop fields and some trees (*Salix babylonica*). Site two, “Sierra Morelos Park” (SMP), is an urban state park of 1,255 hectares managed by the state commission of natural parks and fauna (CEPANAF) since 1976 (44 years); it is immersed in the urban area of Toluca City (19°18'39" N 99°41'33" W; 2,630 m). SMP vegetation is mainly represented by grasslands (*Muhlenbergia* sp. and *Festuca* sp.), and temperate forest vegetation (*Pinus* spp.); it also includes a substantial cover of non-native vegetation, mainly exotic species (buds, *Buddleja cordata*; cedar, *Cupressus lindleyi*; eucalyptus, *Eucalyptus camaldulensis* and *E. globulus*). To eliminate the effect of the size differences between CE and SMP, the sampling area was approximately 20 hectares in both places (Fig. 1).

### Detectability

To determine the detectability of *C. triseriatus*, we performed monthly visits to each study site during 2012 and 2013 (24 visits to each one) and captured individuals of *C. triseriatus*. At each visit, five observers simultaneously walked five linear transects from 0900 to 1300 hours using the visual encounter survey (VES) technique (Foster, 2012). Each transect was approximately 800 m long and separated by 200 to 300 m. Transects may change between visits, but they were always restricted to the 20 hectare area defined for each site, our aim in this regard was to cover all types of vegetation in a similar proportion to their presence. The snakes were manually captured, their snout-to-vent length (SVL) measured, and a ventral scale was cut to identify individuals (Brown & Parker, 1976). Snakes were released in the same place from where they were captured. Additionally, we compared SVL between CE and SMP to test if body size varied between the two sites.

Detectability was determined by counting the number of adult *C. triseriatus* captured during each visit per study site. We recorded data only for adult individuals (SVL ≥ 500 mm) as immature snakes may have led to bias due to the recruitment process (Parpinelli & Marques, 2008; Torello-Viera et al., 2012); as we aim to analyse detectability, we also considered recaptured individuals in this analysis (one individual). We analysed detectability of *C. triseriatus* (response variable) in relation to abiotic (total precipitation, maximum and minimum temperature), and biotic factors (human activity) using general linear models (GLMs) in R version 3.6.3 (R Core Team 2020). For the abiotic factors, we obtained monthly climate data of the climatic variables from the National Meteorological Service database (CLICOM, 2015) for the nearest weather station to CE (< 20 km; weather station “Toluca-Ixtlahuaca”, 19°33'58" N, 99°46'48" W, 2,540 m), and SMP (< 3 km “Toluca-Zinacantepec”, 19°17'29" N, 99°42'52" W; 2,726 m). We also considered the human activity as an important factor to explain snake detectability; for this variable, we characterised both sites based on human presence

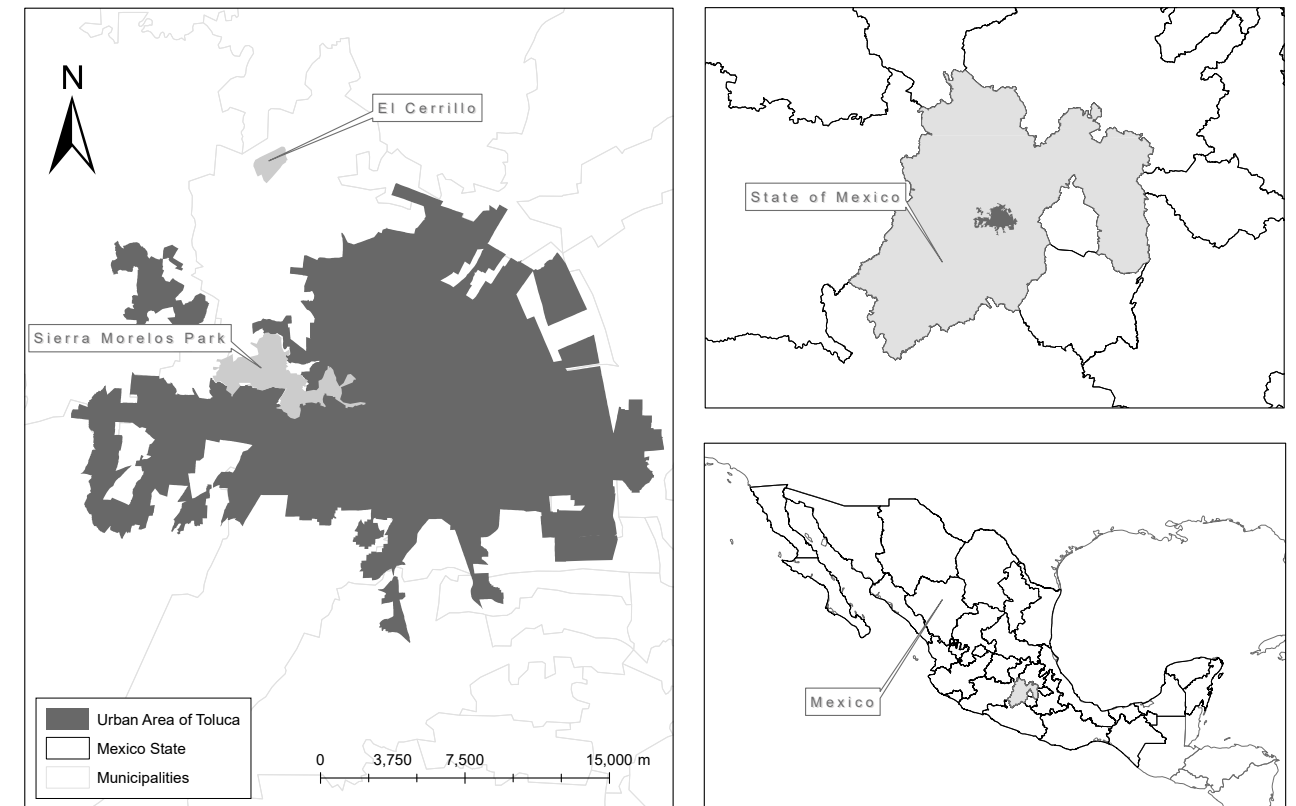


Figure 1. Distribution of the study sites in the urban area of Toluca City, Mexico.

through the year using direct observations at both sites. We codified human activity as absent (0), low human activity (1), and high human activity (2).

Detectability of *C. triseriatus* (number of snakes captured) was assumed to have a Poisson probability distribution and given that our data presented zero values, we used Zero-Inflated Poisson model as a solution for possible bias in the estimated parameters (Zuur et al., 2009), using the “pscl” package (Jackman, 2020). Multiple models were developed using plausible combinations of explanatory variables; small sample Akaike’s information criterion (AICc) was used to select the “best” models of detectability of *C. triseriatus* in relation to explanatory variables, using the ‘AICcmodavg’ package (Burnham & Anderson, 2002; Mazerolle, 2019). Finally, Chi-square tests were conducted to evaluate whether GLM explanatory variables explained a significant component of the total deviance (Guisan et al., 2002).

### Habitat selection

We used satellite imagery (Google Earth) and ArcGIS 10.2 to digitise land cover in both study sites (approximately 20 hectares at each site), and calculated the area represented by native habitat and non-native habitat for each site. Native and non-native habitat cover is noticeably different between our study sites. At CE non-native vegetation represents 87.3 % of the area including crops and exotic trees (*S. babylonica*). Crops are concentrated in four big patches separated by a road bordered with introduced trees. Native vegetation covers 12.7 % of the area and is restricted in two small patches separated by the crops. At SMP native vegetation

represents 58.4 % of the area and is concentrated in a big patch of grassland and another one of forest, both are separated by a road. Non-native vegetation is dispersed in several small patches of exotic trees mostly next to the road.

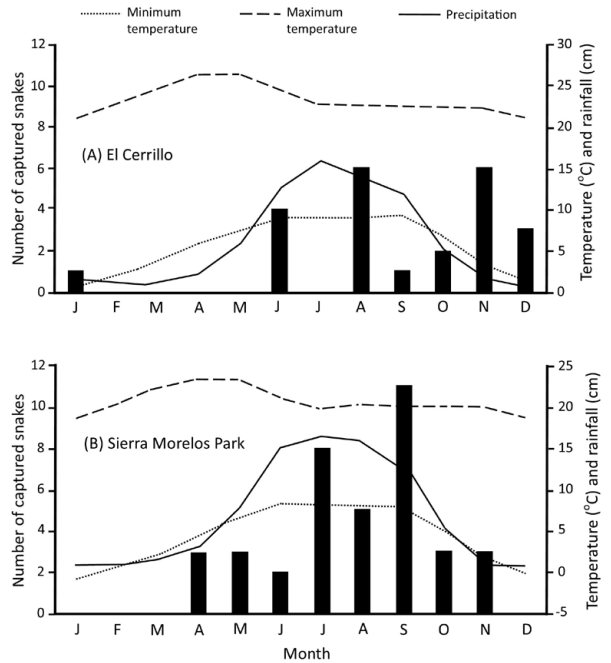
We used the index of selectivity ( $E_i$ ) according to Krebs (1999):  $E_i = (r_i - n_i) / (r_i + n_i)$ , where  $r_i$  is the percentage of snakes in habitat  $i$ ;  $n_i$  is the percentage of habitat  $i$  available at a study site. This index produces values from – 1 (habitat avoidance) to + 1 (habitat preference); values close to zero indicate habitat is being used according to its availability in the environment. The index data were resampled using bootstrap (10,000 replicates with replacement) and 95 % confidence intervals (CI) were estimated with R (v. 3.1.3; Glen et al., 2012).

## RESULTS

During the monthly visits at each study site (CE and SMP), we sampled 192 km of transects and captured 61 different adult *C. triseriatus*; 23 (37.7 %) were from CE, and 38 (62.3 %) from SMP. Snakes from CE were slightly larger ( $605.6 \pm 75.5$  mm, SVL) than SMP ( $589.7 \pm 80.4$  mm). Considering that measurements of the snakes from CE did not show a normal distribution (Shapiro-Wilk test;  $W=0.98$ ,  $P=0.95$ ) and that those from SMP were normally distributed ( $W=0.91$ ,  $P=0.007$ ), we use a non-parametric Mann-Whitney U test (Zar, 1999) and we detected no significant difference in body size between the two sites ( $U=542.5$ ,  $P=0.11$ ).

Detectability

We captured rattlesnakes in all months except February and March at the Toluca valley. Most of the records (61 %), were gathered during the rainy season (June to September). At SMP, 68 % of the snakes were recorded during the rainy season, whilst 48 % were recorded in the same season at CE. Concerning detectability, at CE, we observed two peaks; August (summer) and November (autumn), both months with six snakes captured (Fig. 2A). In contrast, at SMP, snakes presented only one detectability peak in September (11 snakes captured, Fig. 2B). Most snakes were captured from 1000-1100 hours in both sites (CE, 56.5 %; SMP, 52.6 %) regardless of the month.



**Figure 2.** Monthly number of captured rattlesnakes, *C. triseriatus* (bars), monthly precipitation average and temperature (maximum and minimum) at: **(A)** El Cerrillo (CE; high anthropization; n = 23 snakes), and **(B)** Sierra Morelos Park (SMP; low anthropization; n = 38 snakes).

At CE, we observed two peaks of human activity, the first one from February to May, is related to soil preparing before sowing; the second one in September coincides with the harvest period. The rest of the year, the human presence was considered absent. Human activity at SMP is heterogeneous; there is an area where people use to practice sports and recreational activities through the year; at the other portion of the park, human activity is non-allowed because it is considered a conservation reserve for native fauna. Thus, activity was considered very low and homogeneous through the year in the area where we sampled, and we assumed that this factor does not affect (or is too low), the detectability of rattlesnakes. According to the evaluation of GLMs, and based on AIC<sub>c</sub> values, we found that simple single variable models

better explain the detectability of snakes at both sites. At CE human activity explained better the detectability of rattlesnakes (Table 1; AIC<sub>c</sub> = 58.35), in this case, the detectability of *C. triseriatus* decreases when human activity increased (slope estimate=-2.75+1.03; Table 2). This model was significant according to the Chi-square test (sum-of-squares method, type II; X<sup>2</sup>=7.06, df=1, P=0.007). At SMP, based on AIC<sub>c</sub> values, minimum temperature variable explained better the *C. triseriatus* detectability (Table 1; AIC<sub>c</sub> =58.35), this simple GLM model indicates that as the minimum temperature increased, the detectability of the snake also increases (slope estimate=0.30+0.07; Table 2) and this model was also significant (sum-of-squares method, type II; X<sup>2</sup>=17.40, df=1, P=<0.001).

**Table 1.** AIC<sub>c</sub> selection of different zero-inflated Poisson general linear models (GLMs) of *C. triseriatus* detectability in two sites with different levels of anthropization in Mexico; “El Cerrillo” (CE) considered as highly anthropized and the Sierra Morelos Park (SMP) minimally anthropized.

Models of <i>C. triseriatus</i> detectability	K	AIC <sub>c</sub>	ΔAIC <sub>c</sub>
El Cerrillo (CE)			
H_Activ	3	58.35	0.00
H_Activ+TMin	4	60.74	2.38
H_Activ+Prec	4	60.91	2.56
H_ActivXTMax	5	60.99	2.64
H_Activ+TMax	4	61.08	2.73
Sierra Morelos Park (SMP)			
TMin	3	70.11	0.00
TMax+TMin+Prec	5	71.73	1.62
TMax+TMin	4	71.90	1.80
TMin+Year	4	72.06	1.95
TMin+Prec	4	72.81	2.71

Explanatory variable codes: H\_ activ, human activity; TMin, minimum temperature; TMax, maximum temperature; Prec, precipitation; Year, year; K, number of model parameters. The best empirically supported model (ΔAIC<sub>c</sub>=0) in bold.

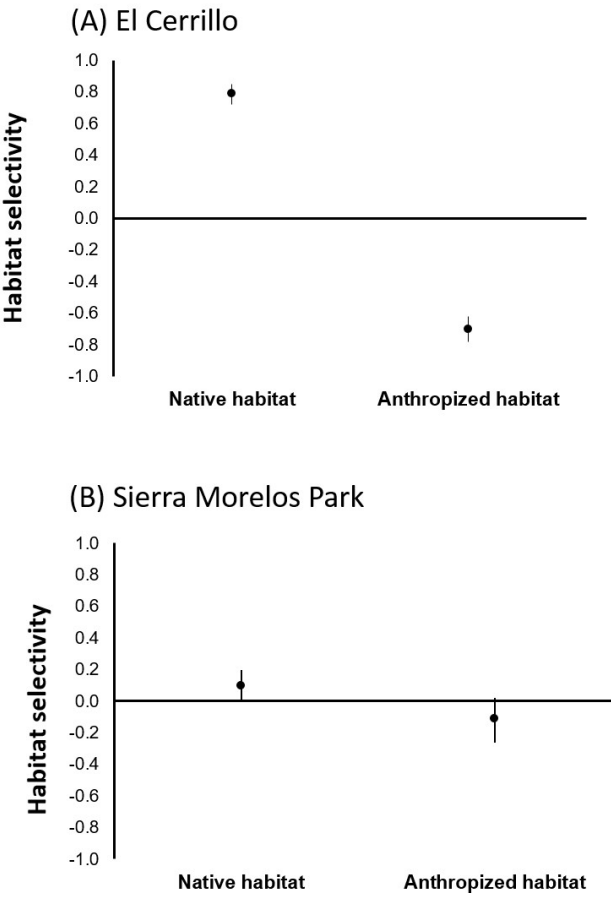
Habitat selection

In both sites, *C. triseriatus* were observed more frequently within the native vegetation compared to the non-native vegetation. For example, at CE, 90 % of snakes were found in the grassland (index of selectivity E<sub>i</sub>= 0.79) and the remaining 10 % in the crop fields (E<sub>i</sub> = -0.70, Fig. 3A). Similarly, at SMP, 82.7 % of snakes were found in native (grassland) habitat (E<sub>i</sub> = 0.10) and 17.3 % in the mixed forest (E<sub>i</sub> = -0.11, Fig. 3B). The index of selectivity indicated that snakes selected native habitat and avoided anthropized habitat at the CE; at SMP snakes showed a qualitatively similar pattern, selecting native habitat and avoiding anthropized habitat (Fig. 3).

**Table 2.** Parameter estimates from the zero-inflated Poisson general linear models (GLM) of effect of human activity (CE) and minimum temperature (SMP) on *C. triseriatus* detectability.

Parameter types/names	Estimate	SE	z value	P value
El Cerrillo (CE)				
Intercept	0.61	0.26	2.34	0.019
H_ activ2	-2.75	1.03	-2.6	0.007
Sierra Morelos Park (SMP)				
Intercept				
TMin	0.30	0.07	-0.09	<0.001

H\_ activ2, high human activity; TMin, minimum temperature; SE, Standard error.



**Figure 3.** Index of selectivity and confidence intervals (C. I.), of *C. triseriatus*, in native and anthropized habitats at: **(A)** El Cerrillo (CE; high anthropization); native habitat E<sub>i</sub> = 0.79 (C. I. 0.72 – 0.85), non-native habitat E<sub>i</sub> = -0.70 (C. I. -0.78, -0.62), and **(B)** Sierra Morelos Park (SMP; low anthropization); native habitat E<sub>i</sub> = 0.1 (C. I. 0.01, 0.20), non-native habitat E<sub>i</sub> = -0.11 (C. I. -0.26, 0.02). Values close to 1.0 indicate habitat preference, and values close to -1.0 indicate habitat avoidance.

DISCUSSION

This study shows that detectability of rattlesnakes in differently anthropized landscapes is associated with different factors. For instance, at CE, human activity was

significantly related with *C. triseriatus* detectability (Table 1; AIC<sub>c</sub>= 58.35) whilst at SMP minimum temperature explained the *C. triseriatus* detectability (Table 1; AIC<sub>c</sub>=58.35). Also, at CE, the detectability of rattlesnakes showed two peaks, one on August and another in November, whilst at SMP, rattlesnakes presented only one peak in September (Fig. 2). Previous studies show that factors derived from anthropization in conjunction with biotic and abiotic factors, influence the activity and hence, detectability of snakes (Moore, 1978; Moreno-Rueda & Pleguezuelos, 2007; BlouinDemers & Weatherhead, 2002; Rocha et al., 2014; Torello-Viera et al., 2012; Weaver, 2008; Wong & Candolin, 2015). According to our results, these factors may be interacting in different ways in the landscapes resulting from anthropic activities and affecting differently the snake populations and maybe other species populations.

Our results indicate that at CE, dynamics of agricultural activities affect the detectability of *C. triseriatus*, producing two monthly peaks. In agricultural areas, the preparation of the soil before sowing starts in February and runs through May, a period that coincided with zero captures of snakes. Later, the presence of snakes increased, coinciding with low agricultural activity during the growth of the crop plants (June – August). Snake captures declined again in September, this coincides with the harvest period. Detectability of *C. triseriatus* increased again with the decline in agricultural activity after maize harvest from October through January (slope estimate = -2.75+1.03; Table 2; Fig. 2).

At SMP we found that minimum temperature is related with the monthly detectability pattern of *C. triseriatus* (slope estimate = 0.30+0.07; Table 2). At SMP, detectability of snakes began to increase in the months of high temperature (April – June). On the other hand in the colder months (December – March), there was a drastic decline in snake detectability, and thus no specimens of *C. triseriatus* were captured at SMP (Fig. 2B), suggesting an inactive period or possible overwinter season at this site, as it has been documented for *Thamnophis scalaris* (Mundo-Hernández et al., 2017). At CE, snakes’ detectability also declined in the colder months; nonetheless, we detected some individuals. The effect of temperature is congruent with previous reports for the species and other rattlesnakes in temperate forests (Heimes, 2016), and is considered a central limiting factor in squamate reptiles in general, as it has been shown that they are particularly dependent on the thermal quality of the environment for thermoregulation (Gibbons & Semlitsch, 1987; Lillywhite, 1987; BlouinDemers & Weatherhead, 2002; Moreno-Rueda & Pleguezuelos, 2007). These results indicate that the snakes at SMP present the same pattern of activity than other wild populations in temperate forests. Otherwise, at CE, the detectability suggests that snakes may be changing its behaviour in response to human activity intensity; Zero-Inflated Poisson GLMs confirm the above (Table 1; AIC<sub>c</sub>= 58.35).

Concerning habitat selection, *C. triseriatus* also showed differences between sites. At CE, the habitat use index shows a strong preference for native habitat



(grassland) and a strong avoidance for anthropized habitats. At SMP native and non-native habitats are used almost according to their availability, we detected a slight avoidance of non-native habitat. Grassland areas are considered an important habitat for this species; the intricate features of the grassland offer foraging opportunities (Matamoros-Trejo & Cervantes, 1992; Mociño-Deloya et al., 2014; Rojas-Martínez et al., 2012) and thermal protection against low temperatures, access to solar energy for thermoregulation, and shelter from predators or humans (Campbell & Lamar, 2004; Cortes-Ávila & Toledo, 2013; Fernández-Badillo et al., 2011). The strong avoidance of anthropized habitat at CE, indicates that agricultural activities may represent a significant negative effect on this species, this is evidenced by the low number of presence records in this habitat even when it represents most of the area (87.3 %). The low availability of native habitat may inhibit movement of snakes in the landscape (Parent & Weatherhead, 2000), leading to a reduction in movement of individuals between populations, and potentially promoting inbreeding. In a genetic study carried out with *C. triseriatus* at Toluca Valley, the gene flow was estimated as moderate but with the possibility of decreasing if the anthropization effects continues to increase (Sunny et al., 2015). Our results also indicate that native vegetation availability is crucial for these snake's persistence as it seems that this habitat may be acting as refugees in the agricultural landscapes. This is evidenced by the great number of presence records on this habitat even when it represents a small portion of the area (12.7 %). Based on this information it seems that conservation studies and future efforts to protect rattlesnakes in anthropized landscapes should consider native habitat management and preservation.

At SMP, *C. triseriatus* used the anthropized and native habitats according to their availability (Fig. 3). However, snakes slightly avoided the use of anthropized habitat. The non-native vegetation presents a high tree cover (>75 %), and the temperature under the canopy of trees may be inadequate for thermoregulation (Lillywhite, 1987). It has been shown that the substrate temperature, more than air temperature, is a determining factor in habitat selection by other species of viperids as *Crotalus viridis*, *C. durissus*, and *Bothrops jararaca* (Gannon & Secoy, 1985; Gomes & Almeida-Santos, 2012). Otherwise, the population at SMP is increasingly confined because there are urban settlements at the periphery of the park. If the park is not properly managed, or if the habitat is altered such that it restricts movement of snakes into and out of the site, problems of inbreeding could potentially occur. Given the limited information about *C. triseriatus* ecology in anthropized sites, the characterisation of its detectability and habitat selection may be crucial for developing effective strategies for control and management of this species. In this study, it is shown that areas with high levels of anthropization (CE) influenced the detectability and habitat selection of *C. triseriatus*. Also, in sites with lower anthropization (SMP), the climatic variables had effects on the detectability of *C. triseriatus* with a slight selection for the native habitat.

Our study shows that *C. triseriatus* presents differences in detectability and habitat selection in anthropized landscapes. Climatic conditions and human activity may be amongst the determinant factors affecting these snakes, and may be other animals, that persist around highly anthropized environments as the urban areas. Nonetheless it is necessary to identify how local differences in the anthropization process (e. g. crop management, landscape structure and composition, human density, and activity intensity) may impact in the response of species. Another important factor to consider is local climate, in this study we used data from the nearest climatic stations to the study locations; nonetheless temperature and humidity may vary at the specific location. Such information may allow for a more precise interpretation of the role of climatic conditions on snake detectability. Also, the influence of intrinsic characteristics of the individuals (e. g. sex and age), must be considered to understand possible variations in the effect of athropization in *C. triseriatus*. Finally, the aversive hunting at both places may affect population size and hence, detectability. We did not recorded any dead snake at our study sites, may be due to access restriction at CE and the conservation status of SMP; nonetheless, we are aware that this is a common practice in the area that need to be studied.

ACKNOWLEDGEMENTS

We want to thank the following: the ‘Comisión Estatal de Parques Naturales y de la Fauna (CEPANAF)’ allowed access to SMP; the organization ‘IDEA WILD’ supported our study with scientific and field equipment for the development of the project; and to Gabriel Varon who contributed to the fieldwork.

REFERENCES

Beaupre, S. J. (1995). Effects of geographically variable thermal environment on bioenergetics of mottled rock rattlesnakes. *Ecology* 76, 1655-1665.

Brown, W. S. & Parker, W. S. (1976). A ventral scale clipping system for permanently marking snakes (Reptilia, Serpentes). *Journal of Herpetology* 10, 247–249.

Brown, W. S., Pyle, D. W., Greene, K. R. & Friedlaender, J. B. (1982). Movements and temperature relationships of timber rattlesnakes (*Crotalus horridus*) in northeastern new york. *Journal of Herpetology* 16, 151-161.

Bryson, R. W., Linkem, C. W., Dorcas, M. E., Lathop, A., Jones, J. M., Alvarado-Díaz, J., Grunwald, C. I. & Murphy, R. W. (2014). Multilocus species delimitation in the *Crotalus triseriatus* species group (serpentes: Viperidae: Crotalinae), with the description of two new species. *Zootaxa* 3826, 475-496.

Burnham, K. P. & Anderson, D. R. (2002). *Model Selection and Multimodel Inference: A Practical Information-Theoretic Approach*. Springer Science & Business Media, New York. 488 p.

Campbell, J. A. & Lamar, W. W. (2004). *The Venomous Reptiles of the Western Hemisphere*. Ithaca Comstock Publishing Associates, New York. 774 p.

Canseco-Márquez, L. & Mendoza-Quijano, F. (2007). *Crotalus*

*triseriatus*. The IUCN Red List of Threatened Species 2007: e.T64338A12771768. Available: <http://dx.doi.org/10.2305/IUCN.UK.2007.RLTS.T64338A12771768.en> [Accessed: 30 August 2018].

CLICOM. (2015). Clima Computarizado. Base de datos. Available: <http://clicom-mex.cicese.mx> [Accessed: 30 August 2018].

COESPO. (2020). Zonas Metropolitanas del Estado de México. [versión electrónica] México. Available: [https://coespo.edomex.gob.mx/zonas\\_metropolitanas](https://coespo.edomex.gob.mx/zonas_metropolitanas) [Accessed: 18 July 2020].

Cortes-Ávila, L. & Toledo, J. J. (2013). Estudio de la diversidad de serpientes en áreas de bosque perturbado y pastizal en San Vicente del Caguán (Caquetá), Colombia. *Actualidades Biológicas* 35, 185-197.

Domínguez-Guerrero, S. F. & Fernández-Badillo, L. (2016). *Crotalus triseriatus* (Mexican Dusky Rattlesnake) Diet. *Herpetological Review* 47, 144-145.

Fernández-Badillo, L., Morales-Capellán, N. & Mayer-Goyenechea, I. G. (2011). *Serpientes Venenosas del Estado de Hidalgo*. México: Universidad Autónoma del Estado de Hidalgo, México. 98 p.

Filippi, E. & Luiselli, L. (2000). Status of the italian snake fauna and assessment of conservation threats. *Biological Conservation* 93, 219-225.

Flores-Villela, O. & Hernández-García, E. (1989). New state records from northern Guerrero, Mexico. *Herpetological Review* 20, 15-16.

Foster MS. 2012. Standard techniques for inventory and monitoring. In McDiarmid RW, Foster MS, Guyer C, Whitfield G and Chernoff N (eds). *Reptile Biodiversity: Standard Methods for Inventory and Monitoring*. Los Angeles/California: Unversity California Press, P. 205-272.

Gannon, V. P. J. & Secoy, D. M. (1985). Seasonal and daily activity patterns in a canadian population of the prairie rattlesnake, *Crotalus viridis viridis*. *Canadian Journal of Zoology* 63, 86-91.

García, E. (2004). Modificaciones al sistema de clasificación climática de Köppen. México: Instituto de Geografía, Universidad Nacional Autónoma de México. 98 p.

Gentili, A. (2004). Italian reptile conservation. *Italian Journal of Zoology* 71, 17-19.

Gibbons, J. & Semlitsch, R. (1987). Activity patterns. Pp. 184-209. In: Snakes: *Ecology and Evolutionary Biology*. Editors, Seigel AR, Collins TJ, Susan NS. New York, McGraw-Hill.

Glen, A. S., Byrom, A. E., Pech, R. P., Cruz, J., Schwab, A., Sweetapple, P. J., Yockney, I., Nugent, G., Coleman, M. & Whitford, J. (2012). Ecology of brushtail possums in a New Zealand dryland ecosystem. *New Zealand Journal of Ecology* 36, 29-37.

Gloor, S., Bontadina, F., Hegglin, D., Deplazes, P. & Breitenmoser, U. (2001). The rise of urban fox populations in Switzerland. *Mammalian Biology* 66, 155–164.

Gomes, C., Almeida-Santos, S. (2012). Microhabitat use by species of the genera *Bothrops* and *Crotalus* (viperidae) in semi-extensive captivity. *Journal of Venomous Animals and Toxins* 18, 393-398.

Gregory, P. T. (1982). Reptilian hibernation. In Biology of the Reptilia, Gans, C. & Pough F. H. Eds. New York Academic Press.

Guisan, A., Edwards, T. C. & Hastie, T. (2002). Generalized linear and generalized additive models in studies of species

distributions: setting the scene. *Ecological Modelling* 157, 89–10.

Güizado-Rodríguez, M. A., Duifhuis-Rivera, C., Maceda-Cruz, R. J., Solano-Zavaleta, I., García-Vázquez, U. O. (2016). Notes on the diet of the Mexican Dusky Rattlesnake, *Crotalus triseriatus* (Viperidae). *Mesoamerican Herpetology* 3, 743–746.

Heimes, P. (2016). *Snakes of Mexico*. Edition Chimaira, Germany. 572p.

Huey, R. B., Peterson, C. R., Arnold, S. J. & Porter, W. P. (1989). Hot rocks and not-so-hot rocks: Retreat-site selection by garter snakes and its thermal consequences. *Ecology* 70, 931-944.

INEGI. (2011). Censo de Población y Vivienda 2010, México. Available: [www.beta.inegi.org.mx/proyectos/ccpv/2010/](http://www.beta.inegi.org.mx/proyectos/ccpv/2010/) [Accessed: 30 August 2018].

Jackman, S. (2020). Political Science Computational Laboratory. R package versión 1.5.5. <https://cran.r-project.org/web/packages/pscl/pscl.pdf>. Accessed 27 April 2020

Krebs, J. C. (1999). *Ecological methodology*. The University of California, California. 620 p.

Lillywhite, H. B. (1987). Temperature, energetics and physiological ecology. Pp. 422-477. In: *Snakes: Ecology and Evolutionary Biology*. Editors, Seige RA, Collins JT, Novak SS. New York, McGraw-Hill.

López-Alcaide, S. & Macip-Ríos, R. (2011). Effects of climate change in amphibians and reptiles. In *Biodiversity Loss in a Changing Planet*, Grillo, O. & Venora, G. Eds. London, IntechOpen.

Madsen, T. & Shine, R. (1996). Seasonal migration of predators and prey a study of pythons and rats in tropical Australia. *Ecology* 77, 149-156.

Manjarrez, J. (2017). Limitación térmica de la actividad en algunos anuros y reptiles como una estrategia ecológica (una revisión). *Ciencia ergo-sum* 71, 78-81.

Marzluff, J. M. & Ewing, K. (2001). Restoration of fragmented landscapes for the conservation of birds: A general framework and specific recommendations for urbaniiing landscapes. *Restoration Ecology* 9, 280-292.

Martins, M. & Oliveira, M. E. (1998). Natural history of snakes in forests of the Manaus region, Central Amazonia, Brazil. *Herpetological Natural History* 6, 78-150.

Matamoros-Trejo, G. J. & Cervantes, F. A. (1992). Alimentos de los roedores *Microtus mexicanus*, *Reithrodontomys megalotis* y *Peromyscus maniculatus* del ex lago de Texcoco, México. *Anales del Instituto de Biología (Serie Zoología)* 63, 135-144.

Mazerolle, M. J. (2019). AICcmodavg: Model selection and multimodel inference based on (Q)AIC(c). R package version 2.2-2. [https:// cran.r-proje ct.org/packa ge=AICcmodavg](https://cran.r-proje ct.org/packa ge=AICcmodavg). Accessed 27 April 2020

Mcdonnell, M. J. & Hahs, A. K. (2015). Adaptation and adaptedness of organisms to urban environments. *Annual Review of Ecology Evolution and Systematics* 46, 261-280.

Mitchell, J. C., Jung, R. E. & Bartholomew, B. (2008). Urban Herpetology. Society for the study of Amphibians and Reptiles, Utah. 608 p.

Mociño-Deloya, E., Setser, K. & Pérez-Ramos, E. (2014). Observations on the diet of *Crotalus triseriatus* (mexican dusky rattlesnake). *Revista Mexicana de Biodiversidad* 85, 1289-1291.

- Moore, R. G. (1978). Seasonal and daily activity patterns and thermoregulation in the southwestern speckled rattlesnake (*Crotalus mitchelli pyrrhus*) and the colorado desert sidewinder (*Crotalus cerastes laterorepens*). *Copeia* 1978, 439-442.
- Moreno-Rueda, G. & Pleguezuelos, J. M. (2007). Long-term and short-term effects of temperature on snake detectability in the wild: a case study with *Malpolon monspessulanus*. *Herpetological Journal* 17, 204-207.
- Mundo-Hernández, V., Domínguez-Vega, H., Gómez-Ortiz, Y., Rubio-Blanco, T., Soria-Díaz, L. & Manjarrez J. (2017). Hibernation refuge of *Thamnophis scalaris* (COPE, 1861), in central Mexico. *Herpetozoa* 29, 198-200.
- Parent, C. & Weatherhead, P. J. (2000). Behavioral and life history responses of eastern massasauga rattlesnakes (*Sistrurus catenatus catenatus*) to human disturbance. *Oecologia* 125, 170-178.
- Parpinelli, L. & Marques, O. A. V. (2008). Seasonal and daily activity in the pale-headed blindsnake *Liotyphlops beui* (serpentes: Anomalepidae) in southeastern brazil. *South American Journal of Herpetology* 3, 207-212.
- Pattishall, A. & Cundall, D. (2009). Habitat use by synurbic watersnakes (*Nerodia sipedon*). *Herpetologica* 65, 183-198.
- Plummer, M. V. (2002). Observations on hibernacula and overwintering ecology of Eastern Hog-nosed snakes (*Heterodon platirhinos*). *Herpetological Review* 33, 89-89.
- Prange, S., Gehrt, S. D. & Wiggers, E. P. (2003). Demographic factors contributing to high raccoon densities in urban landscapes. *Journal of Wildlife Management* 67, 324-333.
- R Core Team (2020) R: A language and environment for statistical computing. R Foundation for Statistical Computing, UK. <https://www.R-project.org/>. Accessed 27 April 2020
- Rocha, M. C., Hartmann, P. A., Winck, G. R. & Cechin, S. Z. (2014). Seasonal, daily activity, and habitat use by three sympatric pit vipers (Serpentes, Viperidae) from southern Brazil. *Anais da Academia Brasileira de Ciências* 86, 695-706.

- Rojas-Martínez, A., Aguilar-López, M., Sánchez-Hernández, C. & Noguera-Cobos, O. (2012). Uso del espacio y dinámica poblacional del ratón de los volcanes (*Neotomodon alstoni*) en el Cerro del Ajusco, Distrito Federal, México. In Estudios sobre la Biología de Roedores Silvestres Mexicanos, Cervantes, F. & Ballesteros-Barrera, C. Eds. México, Universidad Nacional Autónoma de México.
- Sullivan, B. K., Leavitt, D. J. & Sullivan, K. O. (2017). Snake communities on the urban fringe in the sonoran desert: Influences on species richness and abundance. *Urban Ecosystems* 20, 199-206.
- Sunny, A., Monroy-Vilchis, O., Zarco-González, M., Mendoza-Martínez, G. & Martínez-Gómez, D. (2015). Genetic diversity and genetic structure of an endemic mexican dusky rattlesnake (*Crotalus triseriatus*) in a highly modified agricultural landscape: Implications for conservation. *Genetica* 143, 705-716.
- Torello-Viera, N. F., Araújo, D. P. & Braz, H. B. (2012). Annual and daily activity patterns of the snail-eating snake *Dipsas bucephala* (serpentes, dipsadidae) in southeastern brazil. *South American Journal of Herpetology* 7, 252-258.
- Weaver, R. E. (2008). Distribution, abundance, and habitat associations of the night snake (*Hypsiglena torquata*) in washington state. *Northwestern Naturalist* 89, 164-170.
- Wong, B. B. M. & Candolin, U. (2015). Behavioral responses to changing environments. *Behavioral Ecology* 26: 665-673.
- Zar, J. H. (1999). *Biostatistical Analysis*. Prentice-Hall, New Jersey. 663 p.
- Zuur, A. F., Ieno, E. N., Walker, N. J., Saveliev, A. A. & Smith, G. M. (2009). Zero-truncated and zero-inflated models for count data. In: Zuur, A. F., Ieno, E. N., Walker, N. J., Saveliev, A. A. & Smith, G. M. (eds) *Mixed Effects Models and Extensions in Ecology with R*. Springer, New York, pp 261–293

Accepted: 2 January 2021



Published by the British  
Herpetological Society

## Can ventral pattern be used for individual recognition of the vulnerable Pyrenean brook newt (*Calotriton asper*)?

Manon Dalibard<sup>1</sup>, Laëtitia Buisson<sup>1</sup>, Olivier Calvez<sup>2</sup>, Morgan Nguyen-Hong<sup>1</sup>, Audrey Trochet<sup>2,3</sup> & Pascal Laffaille<sup>1</sup>

<sup>1</sup>Laboratoire Ecologie Fonctionnelle et Environnement, Université de Toulouse, CNRS, Toulouse INP, 118 route de Narbonne, 31062 Toulouse Cedex 9, France

<sup>2</sup>Station d'Ecologie Théorique et Expérimentale, UMR 5321, 2 route du CNRS, 09200 Moulis, France

<sup>3</sup>(Present adress) Société Herpétologique de France, Muséum National d'Histoire Naturelle, CP41, 57 rue Cuvier, 75005 Paris

Individual recognition of animal species is a prerequisite for capture-mark-recapture (CMR) studies. For amphibians, photo-identification of body pattern is a non-invasive and less expensive alternative than classical marking methods (e.g. passive integrated transponder). However, photo-identification is effective only if the patterns are (i) sufficiently variable between individuals, and (ii) stable over time. This method also depends on the observer's judgment. In the present study, we assessed the effectiveness of an automatic algorithm (AmphIdent) to recognise ventral colour patterns of the Pyrenean brook newt (*Calotriton asper*), endemic to the Pyrenees Mountains of France. To assess the performance of the tested method, 113 individuals from two different streams were marked with passive integrated transponders (PIT-tags). We used false rejection rate (FRR), false acceptance rate (FAR) and true acceptance rate (TAR) as metrics to evaluate performances of photo-identification. Mean FRR was 7.3 %, FAR was 5.2 %, and TAR was 92 % across both streams, both sexes and all the observers. FAR was significantly different between sexes, while FRR and TAR were significantly influenced by the interaction between the sex and the stream. Despite these differences, our error rates are among the lowest values found in the literature for both amphibian and non-amphibian computer-assisted photo-identification. We found that poor-quality reference pictures could lead to an increasing difficulty to achieve a correct match when time since first capture rose. Consequently, individual photo-identification using AmphIdent software is a reliable tool to aid in the monitoring the Pyrenean brook newts, provided that pictures are taken with care, reference images are regularly updated and observers are properly trained to use the software and interpret images.

**Keywords:** AmphIdent, natural marking, computer recognition, amphibian monitoring, pattern changes

## INTRODUCTION

Capture-mark-recapture (CMR) studies provide important knowledge about demography, life cycles, movements and ecological characteristics of species (Nichols, 1992; Wilson et al., 1999; Honeycutt et al., 2019). Such information is crucial to implement the most appropriate and effective conservation strategies for species or populations (Govindarajulu et al., 2005; Lyet et al., 2008). Capture-mark-recapture studies with amphibians often use invasive techniques to individually mark animals, such as passive integrated transponder (PIT) tagging (Zydlowski et al., 2006; Cucherousset et al., 2008), coloured elastomer subcutaneous marking (Simon, 2007; Josephson et al., 2008) or the archaic method of toe-clipping (Phillott et al., 2007). However, these methods may be of concern due to potential welfare and ethical issues (Narayan et al., 2011). Furthermore, some tags may be lost from animals if the operational mode is not optimal (e.g. anaesthesia and post-operating surveillance) and can affect survival (Reeves & Buckmeier, 2009), growth (Davis & Ovsaka, 2001; Mazel et al., 2013),

and movements (Schmidt & Schwarzkopf, 2010).

A non-invasive alternative to traditional marking techniques is photo-identification. This method relies on natural marking (e.g. spots, stripes, scales or scars) present on animal's body which are compared to an image databank of known individuals. This method is increasingly used in CMR studies to provide reliable demographic data on wildlife populations (Mizroch et al., 2004; Cheney et al., 2014). Identification “by eye” is feasible with a small set of pictures (Silver et al., 2004; Langtimm, 2004). For larger datasets, recent technical advances have enabled the development of photo-matching algorithms of two types: (i) feature-based (detection of distinctive features within the pattern), and (ii) pixel-based (comparison of pixel values between two images) photo-matching algorithms. Photo-matching identification has been used for several taxonomic groups such as mammals (Bolger et al., 2012), reptiles (Sacchi et al., 2010; ), chondrichthyans (Dureuil et al., 2015), osteichthyans (Chaves et al., 2016), insects (Caci et al., 2013; Romiti et al., 2017; Díaz-Calafat et al., 2018) and amphibians (Šukalo et al., 2013; Drechsler et al.,

*Correspondence:* Manon Dalibard (manon.dalibard@toulouse-inp.fr)

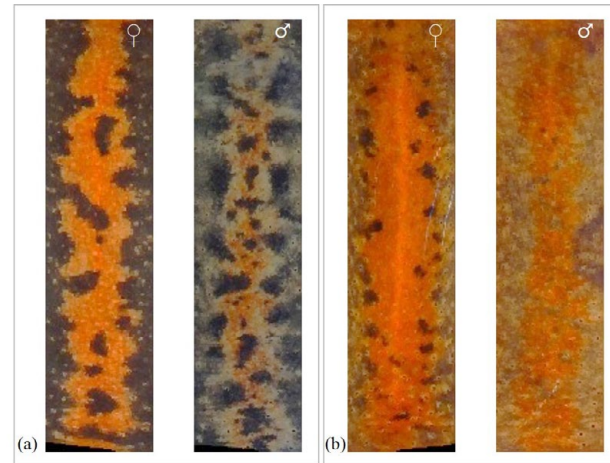


2015; Morrison et al., 2016). In addition to being less invasive for animals, photo-identification method has the advantage of being cheaper and less demanding in materials than traditional marking methods. Its main drawback is the time required to handle animals and to analyse pictures.

Photo-matching identification requires that body patterns are sufficiently variable between individuals and stable enough over time, at least over the study period (Dodd, 2010). These two assumptions are crucial to avoid misidentifications and consequently incorrect estimates of the population parameters (Renet et al., 2019). Moreover, identifying animals through their natural body markings involves a higher risk of subjective assignment than more invasive methods (e.g. reading a PIT tag code). Although the photo-matching software compares a picture with all patterns present in a database to sort them by similarity order, the final diagnostic decision about whether this is a new capture or a recapture indeed comes down to the observer's judgement. Marshall & Pierce (2012) suggested that observer subjectivity is a substantial source of errors in photo-matching studies, while Cruickshank & Schmidt (2017) emphasised a learning effect of the observers in matching identification. However, deviations induced by an observer are seldom considered in studies using computer-aided matching software (Bolger et al., 2012; Cruickshank & Schmidt, 2017).

Most amphibians exhibit natural body marks (e.g. coloured and contrasted patterns, spots) and photo-identification has been successfully applied to several species (e.g. the Jollyville Plateau Salamander *Eurycea tonkawae*, the Iberian midwife toad *Alytes cisternasii*, the marbled salamander *Ambystoma opacum*) (Gamble et al., 2008; Ribeiro & Rebelo, 2011; Bendik et al., 2013). Drechsler et al. (2015) proposed two new promising amphibian candidates to test the effectiveness of photo-matching identification including the Pyrenean brook newt (*Calotriton asper*). This amphibian is endemic to the Pyrenean mountain range (France, Spain and Andorra) and lives in cold and well oxygenated freshwaters (Martínez-Rica & Clergue-Gazeau, 1977; Serra-Cobo, 1989; Arrayago et al., 2005; Montori, et al., 2008; Amat et al., 2011). It is listed in the Appendix IV of the European Council Directive on the Conservation of natural habitats and of wild fauna and flora (Habitats Directive 92/43/CEE, May 21st 1992), in the appendix II of the Berne Convention (JORF of August 28th 1990 and August 20th 1996), and in the national Red List of amphibians of metropolitan France as vulnerable species (IUCN France, 2015). Its conservation suffers from a marked lack of knowledge about its biology and ecology, as well as the factors that influence it directly or indirectly (Dalibard et al., 2020). Population status and trends across its distributional range in the Pyrenees are also poorly known as CMR studies are difficult to implement due to the absence of non-invasive tools to identify individuals. Yet, adults display contrasted black and yellow-orange ventral patterns that could potentially make them good candidates for individual photo-identification (Fig. 1).

In this study, we tested and measured the accuracy of



**Figure 1.** Examples of the ventral patterns of adult female (♀) and male (♂) Pyrenean brook newts, sampled in two streams monitored for the present study: (a) Salau and (b) Fougax (France). The part of the ventral pattern analysed by AmphIdent starts from above the anterior legs and ends at the cloaca.

computer-aided photo-identification for two populations of the Pyrenean brook newt. Specifically, our objectives were: (i) to implement the AmphIdent software for the individual identification of adults of the Pyrenean brook newt, a software specifically targeted to amphibians, (ii) to assess the performance of this software for the Pyrenean brook newt by comparing the results obtained with this method to the monitoring of PIT-tagged individuals and (iii) to measure the ability of AmphIdent to recognise individuals over time.

## MATERIAL AND METHODS

### Field data collection and individual marking

Two populations of Pyrenean brook newt (*Calotriton asper*) were sampled during Summer 2018 in two streams located in Ariège county in France (thereafter named Fougax and Salau). Summer is the period when the species is the most active, which maximises the chance of capturing many individuals (Nicol, 1990). The first sampling was carried out on 4 June 2018 for Fougax (elevation: 700 m) and on 26 June 2018 for Salau (elevation: 1,250 m), in order to mark individuals with electronic PIT-tags. This invasive marking was necessary to validate the pattern recognition by computer-assisted photo-identification. Search for Pyrenean brook newt was conducted by experienced observers from downstream to upstream by looking under rocks and shelters within river-bed, especially riffles and pools, along a 200 m transect within each stream. A total of 59 and 54 adult Pyrenean brook newts were caught in Fougax and Salau, respectively. They were then transported and housed to the Station d'Ecologie Théorique et Expérimentale (SETE, Moulis, France; coordinates: 42°57'29.82"N, 1°05'11.27"E) located about 1 hour drive from both streams. Individuals were kept in captivity for a 48 hour period in 80 x 40 x 35 cm aquariums (maximum 20 animals per aquarium, males and females



**Figure 2.** Photography set up for ventral pattern recognition of the Pyrenean brook newt, located close to the river bank (a and b). Individuals are placed in the glass box placed on a drilled support, between the barrier and the side of the glass box, and covered by a white background (c and d). They are photographed from below with a tripod-mounted camera placed at a standardize distance of 30 cm below the glass box.

were separated). To reflect wild conditions and minimise stress, we kept aquariums at 15 °C and added artificial shelters. After this acclimation period, each animal was placed ventral surface down in a 20 x 10 x 5 cm glass box (with a thin layer of water) to be photographed (Fig. 2). In addition to reduce animal stress, the layer of water minimises water droplets under the animal, which could distort the pattern via a magnifying glass effect. A polystyrene barrier was placed inside the glass box to keep the animal straight. Once the animal was still and straight (from a few seconds to 1-2 min depending on the animal), four pictures of the ventral pattern were taken from below at a standardised distance of 30 cm. Pictures were taken with a camera Nikon Coolpix AW110 © stabilised on a tripod. The camera's flash was always used. Each animal was then anaesthetised by placing a spot of EMLA ointment (5 % Lidocaine 2.5 % and Prilocaine 2.5 %; Astra-Zeneca GmbH Laboratories, Germany, EMLA) in a cutaneous squared surface of 1 x 1 cm, on the left side. Once the animal was considered surgically anaesthetised (i.e. loss of 'withdrawal reflex' and 'righting reflex', Mitchell, 2009), an electronic PIT-tag (Biolog Tiny 10268 – R02-0717 –, tag size: 1.4 x 8 mm, needle size: 1.75 mm; from BIOLOG-ID, FR) was inserted subcutaneously on its left side, between the front and hind legs. Anaesthesia and recovery duration were noted for each animal. Permission for animal marking was issued by DREAL Occitanie (Prefectoral decree n°2017-s-02 from 30 March 2017 to 30 October 2020). Both anaesthesia and PIT-tag marking were conducted during 2 days by a qualified person (user establishment

agreement n° B09583; nominative authorisation n° A09-1) in compliance with ethical standards. Once the animal was awake, it was kept in captivity for 3 or 4 days, depending on the day of marking, to ensure the PIT-tag was not lost and that no post-operative complications occurred. Newts were fed with Tubifex worms ad libitum before and after the anaesthesia and marking. Finally, all captured and marked Pyrenean brook newts were released 7 days after being captured, on 11 June 2018 for Fougax and on 2 July 2018 for Salau, in the 200 m transect where they were captured.

### Recapture of PIT-tagged Pyrenean brook newts

The next step was to sample the same two 200 meter-long transects throughout the summers of 2018 and 2019 in order to recapture PIT-tag marked individuals. Between June and September 2018, we searched for individuals on seven occasions in Fougax (on 21 June, 6 July, 19 July, 2 August, 22 August, 4 September and 28 September) and five occasions in Salau (on 11 July, 26 July, 8 August, 28 August and 11 September), between 0930 and 1400 with a consistent sampling effort (i.e. two samplers for two hours surveying the 200 meter-long transect). Between June and September 2019, we searched for individuals on four occasions in Fougax (on 4 June, 4 July, 2 August and 13 September) and three occasions in Salau (22 July, 8 August and 12 September). Fewer sampling occasions were carried out in Salau than in Fougax, as Salau is inaccessible before July due to a risk of late snowfall episodes. Two samplings of two hours were conducted per occasion, distant in time of at

least one-hour, without animal release between them. Individuals captured were placed in plastic freezer bags. Each animal was photographed four times in the field, using the same material and procedure as in laboratory. To control for varying amount of sunlight when taking pictures, we placed a white background on the top of the glass box (Fig. 2) and we used a sunshade to cover the entire photographing set up in open area (i.e. in Salau). We then scanned each individual for detecting the presence of a PIT-tag and we determined its sex. With two experienced observers to perform these tasks, less than two minutes per individual were required. After manipulation, each individual was replaced in its plastic freezer bag, and stocked in a cool box containing water. Pictures and measures were performed outside the stream, at a standing place on the riverbank quickly accessible from everywhere along the 200 meter-long stream transect (Fig. 2). At the end of the two sampling occasions, all the individuals were released back at the exact location where they were caught. For each sampling occasion, including the initial laboratory marking, a single picture of each individual (the straightest and with the least camera glare) was selected to represent the animal in the database.

Although the two streams studied seem to be uninfected by emerging diseases such as the chytridiomycosis caused by *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorens* or the ranaviruses (Miaud, 2013; Martinez Silvestre et al., 2018; pers. comm.), we minimised as much as possible the risk of disease transmission. All the material used for sampling were disinfected between the sampling occasions by spraying a solution of VIRKON disinfectant (Virkon S powder, concentration: 1 %, time of action: 30 minutes). New freezer plastic bags where captured individuals were stocked during sampling were also used.

Photo-identification with AmphIdent

AmphIdent is an automatic photo-matching software using cross-correlation comparisons and straightening transformation of pictures (Matth   et al., 2008). The first step is to define and extract the pattern zone from the original picture. As the resolution across pictures was the same due to the standardised distance between the glass box containing the animal and the camera, pictures did not need to be resized before the extraction. Thus, less than one minute per picture was required to perform the extraction step. This step consists in adjusting the automatically generated body contour points (i.e. from the location of the anterior legs to the cloaca) into a common rectangular reference space. Second, the algorithm compares the full extracted pattern with all existing images in the reference database. As no spot pattern is exactly the same, even between pictures of the same individual, the algorithm uses transformation on each pixel’s position to transform one pattern into the other. Pairwise comparisons between the extracted pattern and all the patterns in the reference database provide similarity scores, which correspond to the number of matching pixels between the two images. Finally, the observer compares “by eye” the 20 best

images proposed by AmphIdent, which are sorted according to their similarity score, to either (i) choose the matching image (i.e. recapture), or (ii) decide that there is no match in the reference database (i.e. new individual).

Assessment of AmphIdent performances  
Reference image databases and test datasets

Given the dispersal ability of Pyrenean brook newt (in the range of several hundred meters; Montori et al., 2008) and the straight-line distance between the two streams (60 km), we assumed that recaptures were impossible between streams, and thus created one separate reference image database for each stream. Reference image databases (and then test datasets) were separated for males and females given that sex identification is easy in situ and reliable in the Pyrenean brook newt. This categorisation enabled us to limit the number of images in the reference database and thus the computing time. The Fougax reference database includes 59 pictures (22 females, 37 males), and the Salau reference database 54 pictures (22 females, 32 males), which correspond to the pictures taken before PIT-tagging (i.e. one for each marked animal). These four databases are the reference for the photo-matching performance analysis (see Figure 1 for examples of reference pictures).

To assess performances of photo-identification software, rates of false rejection (FRR), false acceptance (FAR) and true acceptance (TAR) are traditionally calculated. False rejection rate is the failure to identify the same individual between two captures whereas false acceptance rate is the incorrect matching between two captures of two different individuals. True acceptance rate is the success of matching the same individual between two captures. To compute these rates, a test dataset was built for each stream and each sex including all the pictures of the PIT-tagged recaptured newts taken during the sampling occasions of summer 2018 (Table 1). The pictures of PIT-tagged recaptured newts give the opportunity to evaluate false rejection errors (i.e. FRR), and the proportion of PIT-tagged recaptured newts which have been correctly matched with AmphIdent (i.e. TAR). A random selection of pictures of Pyrenean brook newts captured during sampling occasions, but not marked with PIT-tags in laboratory, named “unknown”, was added to the test dataset (Table 1). The “unknown” animals are

**Table1.** Number of pictures selected for the four test datasets used for the assessment of AmphIdent performances, two for each sex (males and females) into each stream (Salau and Fougax). For each sex into each stream, PIT-Tagged corresponds to the number of pictures of recaptured newts (i.e. individuals with PIT-tag) and ‘Unknown’ is the number of pictures of individuals without PIT-Tag.

Stream		Salau		Fougax	
Sex	Males	Females	Males	Females	
PIT-Tagged	19	11	46	23	
‘Unknown’	6	3	13	9	
Total	25	14	59	32	

not present in the reference images database and give the opportunity to assess false acceptance errors (i.e. FAR). The number of pictures of “unknown” individuals depended on the number of pictures of PIT-tagged recaptures in the dataset (about 25 % according to Drechsler et al., 2015). The Fougax test dataset included 91 pictures, while the Salau test dataset contained 39 pictures (Table 1).

We differentiated FAR into FAR1 and FAR2, which correspond respectively to (i) the rate of false matching between an animal tested and an “unknown” animal (i.e. there is no match in the reference images database but the observer has assigned one among the “unknown” individuals), and (ii) the rate of false matching between an animal tested and a PIT-tagged animal (i.e. a match exists in the reference images database but the observer has not assigned the good one).

During summer 2018, a total of 41 adult Pyrenean brook newts marked with PIT-tags were recaptured at least once in Fougax and 21 in Salau, representing about 70 and 39 % of marked individuals in each stream, respectively. Among the PIT-tagged recaptured animals, 54 and 67 % were recaptured only once in Fougax and Salau, respectively.

Photo-identification exercise by multiple observers

We asked 10 volunteer observers to implement the photo-identification process using AmphIdent. Observers were scientists and students in zoology, but all inexperienced with photo-identification software. Nine observers were trained in AmphIdent during a course of two hours, where they could perform tests with the software. As the tenth volunteer could not attend the course, he was given an accelerated course before implementing the photo-identification test.

Each observer was asked to compare all the pictures of the four test datasets one-by-one to the corresponding reference images database. To improve matching-recognition, they had to look for a correct match within the top 20 highest-ranking candidate matches. For each picture tested, the observer had to record the unique code and rank (from one to 20) of the image from the reference database matching the best according to them. If the observer found no image from the reference database matching the tested picture, he/she had to record it as well. Time was not limited but the observers were recommended not to spend more than 5 minutes per picture tested. Five minutes is the maximum time we estimated to compare the 20 candidates to the tested picture, even for complex patterns. Thus, recognition effort was standardised, providing against potential bias between observers. The observers were not informed about the “tag” and “unknown” pictures in order to enable identification errors (i.e. FAR and FRR).

Computation of performances metrics

Once all the observers had performed the photo-identification exercise, FRR, FAR (i.e. sum of FAR1 + FAR2) and TAR were computed to assess the performances of AmphIdent. FRR was the number of false rejections (i.e. not recognising a PIT-Tagged individual while it is present

in the test dataset) divided by the number of “PIT-Tagged” pictures in the test dataset. FAR1 was the number of type 1 false acceptances (i.e. assigning a wrong but not PIT-Tagged individual from the test dataset) divided by the number of “unknown” pictures in the test dataset. FAR2 was the number of type 2 false acceptances (i.e. assigning a wrong but PIT-Tagged individual from the test dataset) divided by the number of “PIT-Tagged” pictures in the test dataset. TAR was calculated as the number of true matches (i.e. assigning the correct PIT-Tagged individual from the test dataset) divided by the number of “PIT-Tagged” pictures in the test dataset. These rates (%) were calculated separately for each sex within each stream and for each observer.

The computation of performance metrics was repeated 20 times, each time with a different top k highest-ranking, with k ranging from 1 to 20. For k ranging from 1 to 20, the rank of the best matching picture identified by the observer when examining the top 20 highest-ranking was compared to k. If the rank was greater than k, the criteria “no match” (i.e. false rejection) was assigned to the picture tested. Else, the rank was recorded. For instance, if the observer found a correct match ranked at the 18th position within the top 20 highest-ranking candidates for one picture tested, “no match” was recorded for k ranging from one to 17.

Statistical analyses

To assess the potential effect of the sex and the stream on performance metric values while controlling for the observer effect, we used a linear mixed-effect model (LMM) with the lmer function of the lme4 package of the R software (R Core Team 2018) (Bates et al., 2015). In the model fitted, the response variable was one of the three performance metrics (i.e. FRR, FAR, TAR) while the explanatory variables were the stream and the sex included as fixed effects. The observer was included as a random effect. We tested for the effect of the two explanatory variables and their interaction, using restricted maximum likelihood (REML) estimation method (Bolker et al., 2009). A significance threshold of 0.05 was chosen for all conclusions derived from statistical tests.

Assessment of AmphIdent performance over time  
Study design

We tested the ability of AmphIdent to recognise potential changes in ventral patterns over time in the same two streams as above during a two-year period (from June to September in 2018 and 2019). The pictures of the individual marked with PIT-tag in June 2018 recaptured at least once in summer 2018 and/or in summer 2019 (156 pictures: 113 pictures for Fougax and 43 pictures for Salau), were gathered in a new dataset (hereafter named “time dataset”). A total of 71 unique PIT-Tagged individuals (45 from Fougax and 26 from Salau) was recaptured at least once over the two-year period: 39 % were recaptured once, 28 % were recaptured twice, 17 % were recaptured three times and 15 % were recaptured between four and seven times. We tested the ability of AmphIdent at recognising the PIT-Tagged recaptures of



the time dataset (i.e. recaptures recent or distant from the date of the artificial marking) given that all these PIT-Tagged recaptures correspond to individuals present in the reference image database. If (i) the ventral pattern is sufficiently stable over time, and (ii) AmphIdent and the observer performs well, all the 156 pictures should have a match in the reference database.

The comparison between the pictures of the time dataset and the respective reference image database was done by a single observer, who was the one who had the lowest error rates during the previous described photo-identification exercise. To avoid a potential observer bias, the observer was informed that all pictures of the time dataset had a match in the reference databases.

For each of the 156 pictures of the time dataset, the observer assigned the “1” value if the picture of the animal matched with one of the top 20 ranked images in the reference database (i.e. true acceptance). They also recorded the rank of the matching image. Else the “0” value was assigned (i.e. false rejection).

Statistical analyses

We used a generalised linear mixed model (GLMM) to relate the binary variable (1) true acceptance, (0) false rejection, to the interaction between the time since artificial marking (i.e. with PIT-tags) and the stream, and the interaction between time since artificial marking and the sex of the individual. We controlled for non-independence between several PIT-tagged recaptures of a single individual by including the individual as a random effect in the model. The glmer function of the lme4 library of R was used.

We also tested whether the rank at which PIT-Tagged recaptured individuals were photo-identified (among the top 20 images) increased over time. For this analysis, we kept only the PIT-Tagged recaptured animals for which the image matching was found within the top 20 of the reference database (i.e., “1” value in the previous analysis, 144 pictures). We used a LMM to test the effect of the interaction between the time since artificial marking and the stream, and the interaction between the time since artificial marking and the sex of the individual, on the rank assigned to each picture. As previously, individual was included as random effect to account for several recaptures of a single individual.

RESULTS

Assessment of AmphIdent performances

The proportion of PIT-tagged recaptured newts which have been correctly identified (TAR) with AmphIdent, both sexes combined, was always better when analysing the first 20 images proposed by the software (top 20) than when analysing a lower number of images, with no plateau reached, for both Fougax (nindividuals = 69; nobservers = 10; mean ± SD TAR = 0.89 ± 0.11) and Salau (nindividuals = 30; nobservers = 10, mean (±SD) TAR = 0.95 ± 0.05) (Fig. 3). Consequently, only the metrics results obtained using the top 20 will be shown hereafter. On average among all the observers, streams and sexes (n = 40), false rejection rate (FRR) was 7.3 ± 8.4 % (ranging

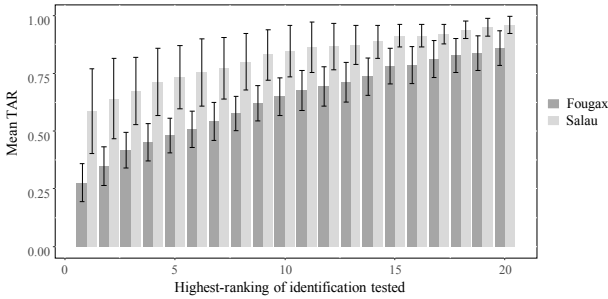


Figure 3. Relationship between the highest-ranking of identification tested and the mean proportion of correct identification (TAR) among all the observers (n=10) for Fougax (dark grey; 69 pictures) and Salau (light grey; 30 pictures). Black bars show the standard deviation of TAR across pictures and observers.

from 0 to 30 %), false acceptance rate (FAR) was 5.2 ± 7.1 % (ranging from 0 to 22 %; mean FAR1 = 4 %; mean FAR2 = 0.8 %), and true acceptance rate (TAR) was 92 ± 9.2 % (ranging from 65 to 100 %).

The interaction between the sex and the stream (Table 2) significantly influenced FRR and TAR. False rejection rate was significantly smaller for females than males in Fougax but there was no difference between sexes in Salau (Table 2). True acceptance rate was significantly higher for females than males in Fougax but there was no difference between sexes in Salau again (Table 2). False rejection rates and true acceptance rates were also significantly different between streams but for males only, with higher FRR and lower TAR values in Fougax than in Salau (Table 2). False acceptance rate varied significantly between sexes, being smaller for females than males when both streams were combined but was similar for the two streams (Table 2).

Table 2. Results of the linear mixed-effect model relating performance metrics to the sex (F: females, M: males) and stream (FOU: Fougax, SAL: Salau): differences of least squares means (means of terms, estimate) with p-values (\* p < 0.10, \*\* p < 0.05, \*\*\* p < 0.01) and standard error (SE), for the FRR, the TAR and the FAR. For each difference term, the estimate is computed from the value of the first part of the difference term. For example, an estimate of 0.031 in the difference term “Sex(F):Stream(SAL) - Sex(M):Stream(SAL)” for the FRR, means that females from Salau has a FRR higher of 0.031 than males from Salau.

Difference Term	FRR	TAR	FAR
	Estimate ± SE	Estimate ± SE	Estimate ± SE
Sex(F) - Sex(M)	-	-	-0.047 ± 0.02*
Stream(FOU) - Stream(SAL)	-	-	0.036 ± 0.02
Sex(F):Stream(FOU) - Sex(M):Stream(FOU)	-0.148 ± 0.02***	0.154 ± 0.02***	-
Sex(F):Stream(SAL) - Sex(M):Stream(SAL)	0.031 ± 0.02	-0.031 ± 0.02	-
Sex(F):Stream(FOU) - Sex(F):Stream(SAL)	-0.038 ± 0.02	0.025 ± 0.02	-
Sex(M):Stream(FOU) - Sex(M):Stream(SAL)	0.141 ± 0.02***	-0.16 ± 0.02***	-

Table 3. Contingency table showing the number of pictures of PIT-tagged recapture from the time dataset for which the match was found in the reference images database (1) or not (0) for both streams and sexes (F : female; M : male).

Stream Sex	Match	Fougax		Salau	
		F	M	F	M
	0	0	7	2	4
	1	38	68	17	20
	Total	38	75	19	24

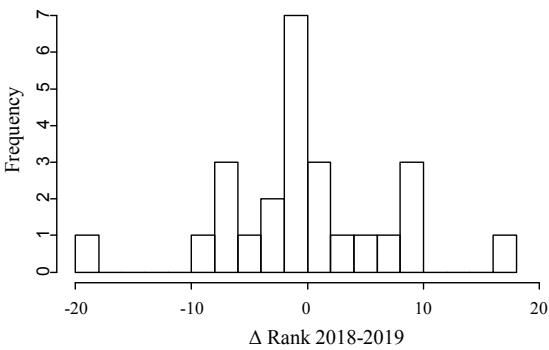


Figure 4. Histogram showing the distribution of the differences between the rank (i.e. position of the identified individual in the top 20 highest-ranking candidate matches) obtained in 2018 and 2019, for individuals recaptured both in 2018 and 2019 (most distant recaptures), all streams and sexes combined. A negative difference in rank indicates that the rank of matching in 2018 was lower than the rank of matching in 2019 for a single animal.

AmphIdent performance over time

For 12 of the 71 Pyrenean brook newts marked with PIT-tags that were recaptured at least once during summer 2018 or summer 2019, one (n = 11) or two (n = 1) pictures of PIT-Tagged recapture were not matched with the images of the reference databases, corresponding to only 13 pictures of the entire time dataset (n = 156; Table 3). However, the GLMM indicated that the time since artificial marking (p = 0.013) and the stream (p = 0.041), significantly influenced the probability of matching with the reference images databases. A negative relationship was found between the time since artificial marking and the probability of matching (slope estimate = -0.004, SE = 0.002, z value = -2.133). The probability of matching was found to be smaller for Pyrenean brook newts recaptured in Salau (estimate = 3.060, SE = 1.225, z value = 2.496) than in Fougax (estimate = 4.180, SE = 1.297, z value = 3.222).

We did not find any evidence that the time since artificial marking has significantly affected the rank at which individuals were photo-identified (p = 0.309). However, the mean rank obtained for Salau (estimate = 3.606, SE = 1.088, t value = 3.313) was significantly lower (p = 0.036) than for Fougax (estimate = 6.328, SE = 0.705, t value = 8.974)

When focusing only on PIT-tagged individuals recaptured both in 2018 and 2019, and comparing the

position (i.e. rank) of a re-identified individual in the top 20 highest-ranking candidate matches between the earliest recapture of 2018 and the latest recapture in 2019, the difference between the positions obtained was not significant (Wilcoxon signed-rank test: p = 0.876; Fig. 4).

DISCUSSION

Photo-identification is an efficient tool in capture-mark-recapture studies and is relevant for many species (Drechsler et al., 2015; Chaves et al., 2016) including protected species with conservation issues (Rowat et al., 2009; Bendik et al., 2013). At present, many automatic software programs are available to assist in wildlife pattern recognition (e.g. APHIS, AmphIdent, I3S, Wild-ID; Moya et al., 2015; Matthé et al., 2008; Bay et al., 2008; Bolger et al., 2012). However, as the platforms use different algorithms, their accuracy can be unequal for a given species (Morrison et al., 2016; Matthé et al., 2017; Renet et al., 2019). Assessing the performance of a photo-identification software for a particular species is thus essential to ensure the quality of a CMR dataset. This study evaluated the performance of AmphIdent software for the individual recognition of adult Pyrenean brook newts. Our results show that AmphIdent provides on average low error rates (false rejection rate = 7.3 %, false acceptance rate = 5.2 %), and a relatively high recognition rate (true acceptance rate = 92 %). These rates are consistent with other studies applying AmphIdent to photo-identify amphibians. For example, Drechsler et al. (2015) reported an FRR of 2 % for the great crested newt *Triturus cristatus*, and Matthé et al. (2017) found that FRR ranged from 0 % to 10.4 % for four species of amphibian. Finally, Goedbloed et al. (2017) found that FRR for the near Eastern fire salamander (*Salamandra atra*) ranged from 0 % with a standardised pictures dataset (i.e. using a standardised photographic procedure, with a consistent distance between the camera and the animal) to 35 % with a non-standardised dataset.

Many studies stress that rising the quality of the pictures can significantly reduce the FRR. For example, Bendik et al. (2013), using another photo-identification software (i.e. Wild-ID), reported a FRR of 15 % for poor-quality pictures and thus concluded that automated photo-identification was not efficient for the Jollyville Plateau salamanders. Using another camera type, they improved the quality of the pictures and decreased greatly the FRR (0.7 %). In the same vein, Morrison et al. (2016) found differences in FRR between two photo-identification software programs (i.e. Wild-ID and Hotspotter) for the Wyoming toad (*Anaxyrus baxteri*), with an FRR of 47 % and 64 %, respectively. They also highlighted that the FRR was improved with higher quality cameras with faster automatic focusing speed. For the photo-identification of the Pyrenean brook newt, we acknowledge that improving the quality of the pictures is an objective readily reachable that would likely reduce error rates (e.g. using portable photography studio to better reduce the variations of ambient

light). In addition, for FRR equalling about 10 %, it is recommended to compare the focal picture to a larger number of pictures (e.g. increased number of pictures to compare to 20, as we did), in order to make sure that the majority of the recaptures can be identified (Chaves et al., 2016; Cruickshank & Schmidt, 2017). Except that comparing more pictures would increase the time needed to analyse the pictures, this recommendation seems also relevant and easy to follow.

Unlike FRR, very few studies using AmphIdent for amphibians have considered FAR and TAR. As far as we know, Drechsler et al. (2015) are the only ones to have computed the FAR for the great crested newt photo-identification, but they found a FAR of 0 % indicating no false acceptance errors. Using other software, FAR is often reported very low, with a maximum value of 1.8 % reported by Bendik et al. (2013) for the Jollyville Plateau salamanders, using Wild-ID. Lastly, TAR ranged between 89.6 % and 100 % in the multi-species study of Matthé et al. (2017) using AmphIdent. This value is also consistent with our results.

In this study, we show that FRR varies significantly between two Pyrenean brook newt populations. Individuals from Salau were indeed easier to recognise than those from Fougax, for both sexes. Our experience with the Pyrenean brook newt suggests that this difference between streams is due to different contrasts in patterns, colour and size of spots (Fig. 1). Pyrenean brook newts from Salau have darker skin than those from Fougax (personal observation). Consequently, contrast with the yellow-orange pattern is more pronounced and could explain why individuals are more easily recognised in Salau (i.e. lower FRR) than in Fougax.

Our results indicate that within a population, the FRR could also be different between sexes. In Fougax, males were significantly harder to recognise than females, which was not the case in Salau (Fig. 1). However, this difference between sexes could mask a difference in the age of the individuals, which was not estimated with accuracy in this study. The Pyrenean brook newt is a long-lived species (>20 years; Clergue-Gazeau, 1971; Montori, 1988) and colour pattern could change over life cycle. Coloration strategies for sexual selection, predation avoidance or thermoregulation are known to change across life stages, in response to changes in competition relationships or environmental conditions (Landová et al., 2013). Thus, if the oldest individuals tended to be either only males or only females in the reference images databases, the difference found between sexes must be interpreted with caution. Finally, as the conspicuousness and contrast of a pattern is dependent on multiple factors, it is difficult to predict the effect of the location, the sex or the age on the performance of individual pattern recognition and the consequences on the estimation of population size, for long-term monitoring programs.

When studying wildlife population dynamics, false negative errors (e.g. FRR) and false positive errors (e.g. FAR) can differently affect inferences about demographic parameters (Royle & Link, 2006; Miller et al., 2011). Using natural markings, Stevick et al. (2001) showed that false negative errors positively bias abundance estimates, as

one unrecognised recaptured individual leads to the creation of a new individual in the reference database. Renet et al. (2019) reported a 3 % over-estimation of population size of the cryptic salamander *Hydromantes strinatii*, with a FRR of 4.3 % (using the top 10 matching pictures with Wild-ID software). For the Pyrenean brook newt, we assume that over-estimation of population size would also likely occur when FRR is high. The larger FRR found for Fougax population could lead to a larger over-estimation of population size or other demographic parameters (e.g. survival rates; Morrison et al., 2011) than in Salau population. Consequently, we recommend estimating the error rates on a larger number of Pyrenean brook newt populations, with a large diversity of ventral patterns, before estimating demographic parameters for this species. We also found a FAR higher (5.2 %) than those found in the literature about amphibians. False positive errors result in large biases when estimating demographic parameters, as they lead to over-estimation of capture probability (Schwartz & Stobo, 1999), due to falsely assigning recaptures to known individuals. False positive error rates are mainly influenced by the observer experience and training (Carlson et al., 1990; Agler, 1992), or the quality of the pictures (Bendik et al., 2013). The relatively high FAR we found suggests that observers must therefore have a good training before being able to use the methodology proposed here.

In this study, we controlled for the observer effect when comparing the FRR, the FAR and the TAR values obtained for each sex and stream. Few studies have explored the possible deviation in error rates caused by the observers, while subjectivity is one of the main acknowledged drawbacks of these methods (Marshall & Pierce, 2012). Cruickshank & Schmidt (2017) compared the performance of photo-identification “by eye” and using a photo-matching software. They emphasised that computer-aided photo-identification reduced the variability in error rates between observers. Cruickshank & Schmidt (2017) highlighted a learning effect in the photo-matching identification, that is, an observer can remember a pattern already encountered, and thus spends less time in the identification process and performs better. This last statement is in accordance with the need to be sufficiently trained using the AmphIdent software and analysing pictures before performing a full CMR study based on natural marking.

One of the most important prerequisites in CMR studies is to have an equal chance to re-identify an individual at all sampling occasions. Using photo-identification methods, this assumption first implies that the body pattern must not change over time. Our results about the performance of AmphIdent to identify Pyrenean brook newts recaptured several months after their first capture revealed that the body patterns were increasingly difficult to match when time elapsed between the first capture and the recapture rose. Even if one individual has already been recaptured and identified once, we cannot thus assume that this individual will be recognised later. Surprisingly, the probability to find a match is lower for Salau than for Fougax, whereas Pyrenean brook newts from Salau are the easiest to identify through their

ventral patterns according to the FRR, FAR and TAR metrics. Pictures that failed to match with the reference images database and particularly those from Salau were of poor quality because of an improper camera’s flash or an inaccurate camera setting, resulting very likely in these identification errors. Consequently, we would recommend that standardised and high-quality pictures are crucial to conduct CMR studies based on individual photo-identification. In the same vein, Mettouris et al. (2016) reported that difference in body conditions over time, such as the weight and gravidity of the individuals, or reproductive status of females, could lead to changes in ranking position during the identification process, without patterns changing. We found one picture from Salau failing to have a match with the reference images database, which was one gravid female at the time of recapture (i.e. with a higher body mass, and subsequently, a distorted ventral pattern). To limit the effect of potentially different body conditions of individuals and the effect of the time elapsed between several recaptures, a solution could be (i) to provide more than one reference image for each individual (but only ventral images as the Pyrenean brook newt has no pattern on the dorsal and lateral sides), and (ii) to regularly update the reference images of the individuals (i.e. each time it is recaptured). Most photo-identification software provide this option, including AmphIdent. Chaves et al. (2016) highlighted that when two reference images of the lionfish *Pterois volitans* were provided, matching probability could reach 100 %. Thus, this solution could substantially improve the performances of photo-identification software such as AmphIdent and reduce error rates.

The robustness of the individual re-identification also depends on the size of the reference database (Matthé et al., 2017). In the present study, we used reference databases of 59 and 54 pictures for Fougax and Salau, respectively, to assess the performances of AmphIdent, that is quite small compared to other studies assessing photo-identification performances in amphibians recognition. Šukalo et al. (2013) used the lowest sample size reported in the literature on amphibians, with 159 individuals of fire salamanders from two populations. Matthé et al. (2017) used much larger databases, with for example 4 063 images of the yellow-bellied toad or 12 488 images of the marbled salamander. However, these databases gathered pictures from many surveys and studies, which does not necessarily reflect the real population size. Matthé et al. (2017) also found that AmphIdent could perform accurately even when increasing the size of the database (i.e. from 500 to 12 488 individuals). This finding was true for the four amphibian species studied. This suggests that AmphIdent could keep good performances in identifying individuals of the Pyrenean brook newt, even with larger databases.

Like many amphibian species, the Pyrenean brook newt requires urgent consideration in conservation strategies, but knowledge about its biology and ecology is lacking (Dalibard et al., 2020). To date, the population dynamics of this species have been very little studied, partly due to the lack of non-invasive methods to identify individuals which is the level required to study

population dynamics. This study emphasises that photo-identification assisted by the AmphIdent software performs well for the Pyrenean brook newt, provided that pictures are taken with care, reference images are regularly updated and observers are trained to use the software and to interpret images of ventral patterns. This method has many advantages compared to more traditional marking methods. First, it makes possible to sample all the Pyrenean brook newts found in the stream, and thus to study population of potentially large size, compared to toe clipping or PIT-tagging which can only be applied to a limited number of individuals due to money or time constraints. Second, the sampling is made to limit stress of newts and handling is limited as much as possible. Third, the material needed to take pictures is very simple, hand-made, light to carry, re-usable and easy to use. Lastly, the total cost is limited to a digital camera and a license for AmphIdent software. Other photo-identification software like Wild-ID or I3S pattern are free to download and use but their performance for the studied species should be assessed before conducting a full CMR study based on photo-identification. The major drawback of the method proposed here is the time required to analyse all the pictures taken in the field (estimated not to exceed 5 to 7 minutes in total for each captured animal, including both the time needed to take pictures in the field and to analyse them with AmphIdent). But this time becomes shorter with experienced observers. Furthermore, as emerging pathogens (e.g. *Batrachochytrium salamandrivorans*) have particularly impacted European newt populations in recent years and pose an important conservation challenge in the Pyrenees (Martinez Silvestre et al., 2018; Dalibard et al., 2020), there is a need to improve the decontamination protocol during sampling occasions (e.g. disinfection of the glass box between each individual, individuals placed in separate plastic bags). Thus, we propose that environmental managers and professionals who manage the territories where the Pyrenean brook newt is present, implement photo-identification method using AmphIdent after a sufficient training, but also within the respect of biosecurity measures to limit pathogens transmission. As long as proper disease-prevention protocols are followed, this would enable them to account for this threatened species in their practices, without deploying oversized and expensive means and within the respect of animal welfare.

#### Conflicts of interest

The authors declare no conflicts of interest

## ACKNOWLEDGEMENTS

We thank all the people who gave time to take part in the assessment of the performances of AmphIdent, as well as Maximilian Matthé for assisting with the software. We thank Bruno Le Roux for his expertise and advice on the Pyrenean brook newt. We also thank warmly all the volunteers and students for their help to capture Pyrenean brook newts from June 2018 to September 2019, in Fougax and Salau: Romain Gadach,



Elisa Gerard, Chloé Dumas, Sylvain Lamothe, Christine Lauzeral, Estelle Ribaut, Franck Gilbert, Frédéric Julien, David Rivas, Alexandre Riberon, Louna Riem, Marilen Haver, Columba Martínez-Espinosa, Marine Deluen, Elodie Darnet, Hugo Le Chevalier, Maxim Lefort, Lauriane Bégué, Alicia Josserand, Pierrick Gaudin.

Finally, we thank EDF (Electricité de France) for their financial support. The project was founded through a PhD contract and by EDF.

## REFERENCES

Agler, B.A. (1992). Testing the reliability of photographic identification of individual fin whales (*Balaenoptera physalus*). *Report International Whaling Commission* 42, 731–737

Amat, F., Sanuy, D. & Palau, A. (2011). Biología del Tritón Pirenaico en el Ibón Acherito. Endesa: Pirineos Centrales.

Arrayago, M. J., Olano, I. & Ruiz de Azua, N. (2005). Censo y caracterización de las poblaciones de tritón pirenaico y sapo de espuelas en la CAPV. Memoria inédita Ekos estudios ambientales S.L-Gobierno Vasco.

Bates, D., Mächler, M., Bolker, B. & Walker, S. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software* 67. DOI:10.18637/jss.v067.i01

Bay, H., Ess, A., Tuytelaars, T. & Van Gool, L. (2008). Speeded-up robust features (SURF). *Computer Vision and Image Understanding* 110, 346-359.

Bendik, N. F., Morrison, T. A., Gluesenkamp, A. G., Sanders, M. S. & O'Donnell, L. J. (2013). Computer-assisted photo identification outperforms visible implant elastomers in an endangered salamander, *Eurycea tonkawae* Ed J. M. Hemmi. *PLoS ONE* 8, e59424. DOI:10.1371/journal.pone.0059424

Bolger, D. T., Morrison, T. A., Vance, B., Lee, D. & Farid, H. (2012). A computer-assisted system for photographic mark-recapture analysis: Software for photographic mark-recapture. *Methods in Ecology and Evolution* 3, 813–822. DOI:10.1111/j.2041-210X.2012.00212.x

Bolger, B. M., Brooks, M. E., Clark, C. J., Geange, S. W., Poulsen, J. R., Stevens, M. H. H. & White, J.-S. S. (2009). Generalized linear mixed models: a practical guide for ecology and evolution. *Trends in Ecology and Evolution* 24, 127–135. DOI:10.1016/j.tree.2008.10.008

Caci, G., Biscaccianti, A. B., Cistrone, L., Bosso, L., Garonna, A. P. & Russo, D. (2013). Spotting the right spot: computer-aided individual identification of the threatened cerambycid beetle *Rosalia alpina*. *Journal of Insect Conservation* 17, 787–795. DOI:10.1007/s10841-013-9561-0

Carlson, C.A., Mayo, C.C. & Whitehead, H. 1990. Changes in the ventral fluke pattern of the humpback whale (*Megaptera novaeangliae*), and its effect on matching: evaluation of its significance to photo-identification research. *Report International Whaling Commission Spec. Issue* 12, 105–111.

Chaves, L. C. T., Hall, J., Feitosa, J. L. L. & Côté, I. M. (2016). Photo-identification as a simple tool for studying invasive lionfish *Pterois volitans* populations. *Journal of Fish Biology* 88, 800–804. DOI:10.1111/jfb.12857

Cheney, B., Corkrey, R., Durban, J. W., Grellier, K., Hammond, P. S., Islas-Villanueva, V., Janik, V. M., Lusseau, S. M., Parsons, K. M., Quick, N. J., Wilson, B. & Thompson, P. M. (2014). Long-term trends in the use of a protected area by small

cetaceans in relation to changes in population status. *Global Ecology and Conservation* 2, 118–128. DOI:10.1016/j.gecco.2014.08.010

Clergue-Gazeau, M. (1971). L'Euprocte pyrénéen, conséquences de la vie cavernicole sur son développement et sa reproduction. *Annales de Spéléologie* 26, 825-960.

Cruikshank, S. S. & Schmidt, B. R. (2017). Error rates and variation between observers are reduced with the use of photographic matching software for capture-recapture studies. *Amphibia-Reptilia* 38, 315–325. DOI:10.1163/15685381-00003112

Cucherousset, J., Marty, P., Pelozuelo, L. & Roussel, J.-M. (2008). Portable PIT detector as a new tool for non-disruptively locating individually tagged amphibians in the field: a case study with Pyrenean brook salamanders (*Calotriton asper*). *Wildlife Research* 35, 780. DOI:10.1071/WR08074

Dalibard, M., Buisson, L., Riberon, A. & Laffaille, P. (2020). Identifying threats to Pyrenean brook newt (*Calotriton asper*) to improve decision making in conservation management: A literature review complemented by expert-driven knowledge. *Journal for Nature Conservation* 54, 125801. DOI:10.1016/j.jnc.2020.125801

Davis, T. M. & Ovaska, K. (2001). Individual recognition of amphibians: Effects of toe clipping and fluorescent tagging on the salamander *Plethodon vehiculum*. *Journal of Herpetology* 35, 217. DOI:10.2307/1566111

Díaz-Calafat, J., Ribas-Marqués, E., Jaume-Ramis, S., Martínez-Núñez, S., Sharapova, A. & Pinya, S. (2018). Individual unique colour patterns of the pronotum of *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae) allow for photographic identification methods (PIM). *Journal of Asia-Pacific Entomology* 21, 519–526. DOI:10.1016/j.aspen.2018.03.002

Dodd, C. K., Jr (2010). Measuring and marking post-metamorphic amphibians. In ‘*Amphibian Ecology and Conservation: A Handbook of Techniques*’ (Ed. Oxford Univ. Press).

Drechsler, A., Helling, T. & Steinfartz, S. (2015). Genetic fingerprinting proves cross-correlated automatic photo-identification of individuals as highly efficient in large capture-mark-recapture studies. *Ecology and Evolution* 5, 141–151. DOI:10.1002/ece3.1340

Dureuil, M., Towner, A., Ciolfi, L. & Beck, L. (2015). A computer-aided framework for subsurface identification of white shark pigment patterns. *African Journal of Marine Science* 37, 363–371. DOI:10.2989/1814232X.2015.1077888

Gamble, L., Ravela, S. & McGarigal, K. (2007). Multi-scale features for identifying individuals in large biological databases: an application of pattern recognition technology to the marbled salamander *Ambystoma opacum*. *Journal of Applied Ecology* 45, 170–180. DOI:10.1111/j.1365-2664.2007.01368.x

Goedbloed, J. D., Segev, O., Küpfer, E., Pietzsch, N., Matthe, M. & Steinfartz, S. (2017). Evaluation of a new Amphident module and sources of automated photo identification errors using data from *Salamandra infraimmaculata*. *Salamandra* 53, 314-318.

Govindarajulu, P., Altwegg, R. & Anholt, B. R. (2005). Matrix model investigation of invasive species control: bullfrogs on Vancouver island. *Ecological Applications* 15, 2161–2170. DOI:10.1890/05-0486

Honeycutt, R. K., Garwood, J. M., Lowe, W. H. & Hossack, B.

R. (2019). Spatial capture–recapture reveals age- and sex-specific survival and movement in stream amphibians. *Oecologia* 190, 821–833. DOI:10.1007/s00442-019-04464-3

IUCN France, MNHN & SHF (2015). La Liste rouge des espèces menacées en France –Chapitre Reptiles et Amphibiens de France métropolitaine. Paris, France.

Josephson, D. C., Robinson, J. M., Weidel, B. C. & Kraft, C. E. (2008). Long-term retention and visibility of visible implant elastomer tags in brook trout. *North American Journal of Fisheries Management* 28, 1758–1761. DOI:10.1577/M08-019.1

Landová, E., Jančúchová-Lásková, J., Musilová, V., Kadochová, Š. & Frynta, D. (2013). Ontogenetic switch between alternative antipredatory strategies in the leopard gecko (*Eublepharis macularius*): defensive threat versus escape. *Behavioral Ecology and Sociobiology* 67, 1113–1122. DOI:10.1007/s00265-013-1536-3

Langtimm, C. A., Beck, C. A., Edwards, H. H., Fick-Child, K. J., Ackerman, B. B., Barton, S. L. & Hartley, W. C. (2004). Survival estimates for Florida manatees from the photo-identification of individuals. *Marine Mammal Science* 20, 438–463. DOI:10.1111/j.1748-7692.2004.tb01171.x

Lyet, A., Cheylan, M., Prodon, R. & Besnard, A. (2009). Prescribed fire and conservation of a threatened mountain grassland specialist: a capture-recapture study on the Orsini’s viper in the French alps. *Animal Conservation* 12, 238–248. DOI:10.1111/j.1469-1795.2009.00245.x

Marshall, A. D. & Pierce, S. J. (2012). The use and abuse of photographic identification in sharks and rays. *Journal of Fish Biology* 80, 1361–1379. DOI:10.1111/j.1095-8649.2012.03244.x

Martínez-Rica, J. P. & Clergue-Gazeau, M. (1977). Données nouvelles sur la répartition géographique de l'espèce *Euproctus asper* (Duges) Urodele Salamandridae. *Bulletin de La Société D'histoire Naturelle de Toulouse* 113, 318–330.

Matthé, M., Schönbrodt, T. & Berger, G. (2008). Computergestützte Bildanalyse von Bauchfleckensmustern des Kammolchs (*Triturus cristatus*). *Zeitschrift für Feldherpetologie* 15(1), 89–94.

Matthé, M., Sannolo, M., Winiarski, K., Spitzen - van der Sluijs, A., Goedbloed, D., Steinfartz, S. & Stachow, U. (2017). Comparison of photo-matching algorithms commonly used for photographic capture-recapture studies. *Ecology and Evolution* 7, 5861–5872. DOI:10.1002/ece3.3140

Martinez Silvestre, A., Verdaguer Foz, I., Trochet, A. & Calvez, O. (2018). Detection and monitoring of *Batrachochytrium dendrobatidis* in the Pyrenean brook salamander (*Calotriton asper*). September Conference at the XV Congreso Luso Español De Herpetologia.

Mazel, V., Charrier, F., Legault, A. & Laffaille, P. (2013). Long-term effects of passive integrated transponder tagging (PIT tags) on the growth of the yellow European eel (*Anguilla anguilla* (Linnaeus, 1758). *Journal of Applied Ichthyology* 29, 906–908. DOI:10.1111/jai.12111

Mettouris, O., Megremis, G. & Giokas, S. (2016). A newt does not change its spots: using pattern mapping for the identification of individuals in large populations of newt species. *Ecological Research* 31, 483–489. DOI:10.1007/s11284-016-1346-y

Miaud, C. (2013). *Batrachochytridium dendrobatidis* chez les Amphibiens des Pyrénées Orientales: Bilan des études

sur la prévalence dans les Réserves naturelles catalanes. Internal report of the Fédération des Réserves Catalanes.

Miller, D. A., Nichols, J. D., McClintock, B. T., Grant, E. H. C., Bailey, L. L. & Weir, L. A. (2011). Improving occupancy estimation when two types of observational error occur: non-detection and species misidentification. *Ecology* 92, 1422–1428. DOI:10.1890/10-1396.1

Mitchell, M. A. (2009). Anesthetic considerations for amphibians. *Journal of Exotic Pet Medicine* 18, 40–49. DOI:10.1053/j.jepm.2008.11.006

Mizroch, S. A., Herman, L. M., Straley, J. M., Glockner-Ferrari, D. A., Jurasz, C., Darling, J., Cerchio, S., Gabriele, C. M., Salden, D. R. & Ziegesar, O. (2004). Estimating the adult survival rate of Central North Pacific humpback whales (*Megaptera novaeangliae*). *Journal of Mammalogy* 85, 963–972. DOI:10.1644/BOS-123

Montori, A., Llorente, G. & Richter-Boix, À. (2008). Habitat features affecting the small-scale distribution and longitudinal migration patterns of *Calotriton asper* in a Pre-Pyrenean population. *Amphibia-Reptilia* 29, 371–381. DOI:10.1163/156853808785112048

Montori, A. (1988). Estudio sobre la biología y ecología del tritón pirenaico *Euproctus asper* (Duges, 1852) en La Cerdanya. Tesis Doctoral. Universitat de Barcelona.

Morrison, T. A., Keinath, D., Estes-Zumpf, W., Crall, J. P. & Stewart, C. V. (2016). Individual identification of the endangered Wyoming toad *Anaxyrus baxteri* and implications for monitoring species recovery. *Journal of Herpetology* 50, 44–49. DOI:10.1670/14-155

Morrison, T. A., Yoshizaki, J., Nichols, J. D. & Bolger, D. T. (2011). Estimating survival in photographic capture-recapture studies: overcoming misidentification error. *Methods in Ecology and Evolution* 2, 454–463. DOI:10.1111/j.2041-210X.2011.00106.x

Moya, Ó., Mansilla, P.-L., Madrazo, S., Igual, J.-M., Rotger, A., Romano, A. & Tavecchia, G. (2015). APHIS: A new software for photo-matching in ecological studies. *Ecological Informatics* 27, 64–70. DOI:10.1016/j.ecoinf.2015.03.003

Narayan, E. J., Molinia, F. C., Kindermann, C., Cockrem, J. F. & Hero, J.-M. (2011). Urinary corticosterone responses to capture and toe-clipping in the cane toad (*Rhinella marina*) indicate that toe-clipping is a stressor for amphibians. *General and Comparative Endocrinology* 174, 238–245. DOI:10.1016/j.ygcen.2011.09.004

Nichols, J. D. (1992). Capture-recapture models. *BioScience* 42, 94–102. DOI:10.2307/1311650

Nicol, A. (1990). L'Euprocte des Pyrénées *Euproctus asper asper* (Duges 1852). Arudy, France.

Phillott, A. D., Skerratt, L. F., McDonald, K. R., Lemckert, F. L., Hines, H. B., Clarke, J. M. & Speare, R. (2007). Toe-clipping as an acceptable method of identifying individual anurans in mark recapture studies. *Herpetological Review* 38, 305-308. DOI:10.1038/srep39739

R Core Team (2018). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Reeves, K. S. & Buckmeier, D. L. (2008). Mortality, predation, and tag visibility of fish marked with visible implant elastomer tags. *North American Journal of Fisheries Management* 29, 323–329. DOI:10.1577/M08-086.1

Renet, J., Leprêtre L., Champagnon J. & Lambret P. (2019).

Monitoring amphibian species with complex chromatophore patterns: a non-invasive approach with an evaluation of software effectiveness and reliability. *Herpetological Journal* 29(1), 13–22. DOI:10.33256/hj29.1.1322

Ribeiro, J. & Rebelo, R. (2011). Survival of *Alytes cisternasii* tadpoles in stream pools: a capture-recapture study using photo-identification. *Amphibia-Reptilia* 32, 365–374. DOI:10.1163/017353711X584186

Romiti, F., Bissattini, A. M., Buono, V., Cifarelli, C., Della Rocca, F., Eniang, E. A., Akani, G. C., Luiselli, L., Superti, V., Carpaneto, G. M. & Vignoli, L. (2017). Photographic identification method (PIM) using natural body marks: A simple tool to make a long story short. *Zoologischer Anzeiger* 266, 136–147. DOI:10.1016/j.jcz.2016.11.006

Rowat, D., Speed, C. W., Meekan, M. G., Gore, M. A. & Bradshaw, C. J. A. (2009). Population abundance and apparent survival of the vulnerable whale shark *Rhincodon typus* in the Seychelles aggregation. *Oryx* 43, 591. DOI:10.1017/S0030605309990408

Royle, J. A. & Link, W. A. (2006). Generalized site occupancy models allowing for false positive and false negative errors. *Ecology* 87, 835–841. DOI:10.1890/0012-9658(2006)87[835:GSOMAF]2.0.CO;2

Sacchi, R., Scali, S., Pellitteri-Rosa, D., Pupin, F., Gentili, A., Tettamanti, S., Maiocchi, V., Racina, L., Caviglioli, M., Galeotti, P. & L., Fasola (2010). Photographic identification in reptiles: a matter of scales. *Amphibia-Reptilia* 31, 489–502. DOI:10.1163/017353710X521546

Schmidt, K. & Schwarzkopf, L. (2010). Visible implant elastomer tagging and toe-clipping: effects of marking on locomotor performance of frogs and skinks. *The Herpetological Journal* 20(2), 99-105.

Schwartz, C.J. & Stobo, W.T. (1999). Estimation and effects of tag-misread rates in capture–recapture studies. *Canadian Journal of Fisheries and Aquatic Sciences* 56, 551–559.

Serra-Cobo, J. (1989). Presencia del tritón melánico (*Euproctus asper*) en el parque nacional de Ordesa y Monte Perdido. Lucas Mallada. *Revista de Ciencias* 0(1), 203–205.

Silver, S. C., Ostro, L. E. T., Marsh, L. K., Maffei, L., Noss, A. J., Kelly, M. J., Wallace, R. B., Gómez, H. & Ayala, G. (2004). The use of camera traps for estimating jaguar *Panthera onca* abundance and density using capture/recapture analysis. *Oryx* 38, 148–154. DOI:10.1017/S0030605304000286

Simon, J. (2007). Evaluation of marking European silver eels with visible implant elastomer tags and alcian blue. *Journal of Fish Biology* 70, 303–309. DOI:10.1111/j.1095-8649.2006.01260.x

Stevick, P., Palsboll, P.J., Smith, T.D., Bravington, M.V. & Hammond, P.S. (2001). Errors in identification using natural markings: rates, sources, and effects on capture–recapture estimates of abundance. *Canadian Journal of Fisheries and Aquatic Sciences* 58, 1861–1870.

Šukalo, G., Đorđević, S., Golub, D., Dmitrović, D. & Tomović, L. (2013). Novel, non-invasive method for distinguishing the individuals of the fire salamander (*Salamandra salamandra*) in capture-mark-recapture studies. *Acta Herpetologica* 8(1), 41-45.

Wilson, B., Hammond, P. S. & Thompson, P. M. (1999). Estimating size and assessing trends in a coastal bottlenose dolphin population. *Ecological Applications* 9, 288–300. DOI:10.1890/1051-0761(1999)009[0288:ESAATI]2.0.CO;2

Zydlowski, G. B., Horton, G., Dubreuil, T., Letcher, B., Casey, S. & Zydlowski, J. (2006). Remote monitoring of fish in small streams: A unified approach using PIT tags. *Fisheries* 31, 492–502. DOI:10.1577/1548-8446(2006)31[492:RMOFIS]2.0.CO;2

Accepted: 11 January 2021



Published by the British Herpetological Society

<https://doi.org/10.33256/31.2.111117>

# How reliable is the habitat suitability index as a predictor of great crested newt presence or absence?

Andrew S. Buxton<sup>1,2</sup>, Hannah Tracey<sup>3</sup> & Nick C. Downs<sup>3,4</sup>

<sup>1</sup> Durrell Institute of Conservation and Ecology, School of Anthropology and Conservation, University of Kent, Marlowe Building, Canterbury, Kent, CT2 7NR, UK

<sup>2</sup> Amphibian and Reptile Conservation Trust, 744 Christchurch Road, Boscombe, Bournemouth, Dorset, BH7 6BZ, UK

<sup>3</sup> Arcadis, 2 Glass Wharf, Bristol, BS2 0FR, UK

<sup>4</sup> University of Bristol, School of Biological Sciences, Woodland Road, Bristol, BS8 1UG, UK

The application of a habitat suitability index (HSI) assessment to predict the use of ponds by great crested newts (*Triturus cristatus*) is commonly used in association with distribution and monitoring projects. Such projects are often used to inform development and planning decision making. However, this type of assessment is frequently misused, and misinterpreted. We used a large, commercially collected environmental DNA (eDNA) survey for great crested newt pond occupancy (489 ponds) to; (1) assess whether it is appropriate to use low HSI scores to rule out occupancy, (2) discuss the use of high HSI scores to identify ponds of high importance for the species and, (3) explore the eDNA detection method. We conclude that there is no evidence to support ruling out pond occupancy based on low HSI scores. However, the conventional view that ponds with HSI scores above 0.7 are of high importance to great crested newts is somewhat supported by the data. Both eDNA and direct observational survey methodologies suffer from sampling error and these need to be acknowledged in the analysis of large data sets.

**Keywords:** Great crested newt, habitat suitability index, HSI, *Triturus cristatus*, environmental DNA, presence absence survey

Mechanisms for quickly estimating the suitability of habitat for a particular species in the field are commonplace in ecology. These can be simply based on expert opinion or formalised via standardised assessments such as habitat suitability indexes (U.S. Fish and Wildlife Service, 1981). However, data from such assessments can be difficult to interpret which can lead to misuse.

Great or Northern crested newts (*Triturus cristatus*) are the most widespread of the *Triturus* super-species consisting of seven closely related species, with a distribution from the UK and France in western Europe across to the Ukraine and southern Russia in the East (Wielstra et al., 2019). Within the UK, great crested newts are the largest of the three native newt species and occupy a wide variety of habitats. These include lowland river valleys with spring-fed ponds which do not flood (Inns, 2009), brownfield sites (Baker et al., 2011), broadleaved or mixed woodland, undisturbed grassland (Jehle & Arntzen, 2000; Skei et al., 2006), and urban fringe (Harper et al., 2019) as well as other habitat types. As a semi aquatic amphibian both aquatic and terrestrial habitat quality is highly important in supporting a viable population, a factor which is often overlooked.

Within Europe, the species is protected by various national and international legal instruments including the

Convention on the Conservation of European Wildlife and Natural Habitats 1979, and the Conservation of Habitats and Species (Amendment) (EU Exit) Regulations 2019. Within the UK, the species is listed under the Conservation of Habitats and Species Regulations 2017 (as amended), the Wildlife and Countryside Act 1981 (as amended) in England and Wales, and the Conservation (Natural Habitats, &c.) Amendment (Scotland) Regulations 2019 in Scotland. This legislation means that a greater level of effort is applied to distribution assessments for the great crested newt than most species. Assessments of impact, for example for land use change, are required (English Nature, 2001), while national assessments of the species distribution and conservation status also require regular reporting.

Habitat suitability assessments have been used for decades, with many developed for a wide variety of species in the 1980s (U.S. Fish and Wildlife Service, 1976, 1980, 1981), some examples being for the muskrat (*Ondatra zibethicus*) (Allen & Hoffman, 1984) and the Brown Trout (*Salmo trutta*) (Wesche et al., 1987). The great crested newt Habitat Suitability Index (HSI) was developed by Oldham et al. (2000) to assess the potential quality of a pond for great crested newts. It has been amended and simplified since (ARG UK, 2010). Although it was first developed for use in the UK, it has

Correspondence: Andrew Buxton (A.S.Buxton@kent.ac.uk)



since been used widely across Europe (Unglaub et al., 2015). The assessment involves the rapid categorisation of the habitat using a number of variables, with minimal equipment, and interpretation can vary widely between individual surveyors. Additionally, the final calculation treats each variable with equal weighting, an assumption that is unlikely to reflect the ecological importance of the different variables. Interpretation of the HSI therefore needs to be treated with a certain level of caution. The great crested newt HSI is used by some practitioners as an indicator of newt presence or absence: this is potentially a misinterpretation of its predictive power.

It is a requirement within the UK to undertake an HSI assessment alongside any great crested newt surveys where the results will be used to inform planning conditions. However, it is clear that the HSI should not replace direct surveys for the species (Natural England, 2015). HSI assessments are key components of both the national monitoring programme (Pondnet, 2013), and the various monitoring schemes for recently launched District Level Licencing (DLL) approaches to great crested newt conservation (Burgess, 2020; Nature Space Partnership, 2019). Nonetheless, the way in which the HSI is interpreted in these settings is not always robust or supported by evidence. In terms of habitat creation, an arbitrary HSI score of 0.7 has been assigned to generally indicate ‘success’. Instances of using HSI scores that would be classed as ‘Poor’ or in some instances ‘Below Average’ as a justification to rule out further commercial survey work, have been brought to the attention of the authors.

In recent years surveys targeting environmental DNA (eDNA) have been developed as a cost-effective and rapid tool for the assessment of great crested newt pond occupancy (Biggs et al., 2014, 2015; Buxton et al., 2017; Rees et al., 2014). eDNA surveys target DNA that has been shed by the target organism and become suspended in the water column (Harper et al., 2018; Jane et al., 2015; Thomsen & Willerslev, 2015). eDNA surveys have advantages over direct observational survey methods, in that they require only a single visit to a pond (Biggs et al., 2015). Surveys can be undertaken as lone workers and in daylight hours. Direct observational methodologies require several overnight visits using multiple methodologies and teams of people, to obtain a similar detection rate (Buxton et al., 2018; Sewell et al., 2010). As a result, eDNA analysis enables large-scale distribution assessments to be conducted with relative ease which were not previously possible (Biggs et al., 2015).

We examine whether the HSI is a good predictor of species occupancy, using a large-scale distribution assessment of the great crested newt in north-west England. We analyse the distribution of HSI results obtained for ponds occupied by great crested newts alongside those with no confirmed occupancy, and discuss the utility of the technique as a fine-scale predictor of species presence-absence. We examine the individual suitability index variables to determine the relative importance of each within the prediction of pond occupancy. We also make conclusions about the applicability of the eDNA-based occupancy information to large-scale commercial distribution assessments.

METHODS

Site selection and protocols

The ponds were surveyed as part of the ecological assessment process for the National Grid North West Coast Connections project, which aimed to install approximately 180 km of new powerline roughly parallel to the Cumbrian (UK) coastline. All ponds surveyed were within the linear area expected to be directly impacted by works (the order limits), plus a 500 m buffer either side. Ponds were initially identified via a detailed review of Ordnance Survey maps and high-resolution aerial imagery. The presence of these ponds in the field was checked during individual pond HSI surveys. Additional ponds were discovered from phase 1 habitat surveys of the entire order limits and 500 m buffer area. In total, 489 ponds were surveyed.

All field surveys adhered to strict biosecurity measures, following guidance from ARG UK (ARG UK, 2017). This comprised either using equipment for one site only, or cleaning, disinfecting and drying between sites. Footwear and vehicle wheels were also disinfected and dried between sites. In all cases, a disinfectant solution of Virkon® was used.

eDNA surveys

eDNA samples were collected from 464 ponds using sampling methodologies adopted for commercial analysis within the UK, as regulated by Natural England and described in Biggs et al. (2014). eDNA sample collection was undertaken during the great crested newt breeding season in 2015, 2016 or 2017. Each pond was sampled at twenty locations around the edge, where 30 mL of water were collected using a dipper and transferred to a Whirl-Pak® self-standing sterile plastic bag. The composite sample from around the pond was homogenised and then subsampled to preserve 15 mL of sample in each of six 50 mL centrifuge tubes containing 1.5 mL of 3M sodium acetate solution and 33 mL of 99 % ethanol. The samples were then analysed using quantitative PCR (qPCR) in a commercial laboratory following the methodology laid out in Biggs et al. (2015), using PCR primers and hydrolysis probe developed by Thomsen et al. (2012). All samples were assessed for both degradation and PCR inhibition using an internal positive control DNA introduced to both sample collection tubes prior to field collection, and at the qPCR stage.

Complexities associated with the survey schedule (for example land access restrictions or changing survey boundaries) resulted in direct observational surveys of 25 ponds without prior eDNA surveys.

Direct observational surveys

A subset of 103 ponds were subject to direct observational survey methods in addition to eDNA sampling, while 85 ponds were either surveyed using observational methods only or returned an inclusive eDNA survey result which was followed up with observational surveys. In addition to result confirmation, direct observational surveys were undertaken to obtain a population size class estimate (English Nature, 2001). A combination of bottle trapping, torchlight counts, hand searches for eggs and sweep

netting for adults was undertaken following standard commercial methodologies for the UK (Cresswell & Whitworth, 2004; English Nature, 2001; Griffiths & Inns, 1998; Griffiths et al., 1996; Langton et al., 2001; Sewell et al., 2013).

Habitat Suitability Index

A HSI assessment was undertaken on each of the 489 ponds (Oldham et al., 2000). In total, ten habitat variables are recorded in the field, comprising geographic location, pond area, frequency of drying, water quality, pond shading, waterfowl presence, fish presence, pond density, the composition of the immediate terrestrial habitat and pond macrophyte cover. Each of these categories are assigned a value of between 0.01 and 1.0 with the geometric mean of the ten values taken to estimate the HSI of a pond for great crested newts. Overall HSI scores close to zero represent unsuitable habitat, while those closer to one represent optimal habitat (Unglaub et al., 2015). HSI categories were introduced by ARG UK advice note (ARG UK, 2010) as a way to help the interpretation of results, the categories were split so that approximately 20 % of ponds fall within each of the five categories. These categories are ‘Poor’ (HSI = 0-0.49), ‘Below Average’ (HSI=0.5-0.59), ‘Average’ (HSI=0.6-0.69), ‘Good’ (HSI=0.7-0.79) and ‘Excellent’ (HSI=0.8-1).

Analysis

Data from all occupancy assessment methods was pooled to generate naïve estimates for occupancy, with simple percentages used to compare occupancy when both eDNA and observational surveys had been conducted on the same water bodies. We compare the proportions of occupied and unoccupied ponds falling into each of the HSI categories.

We used a general linear model to assess whether there is a statistically significant difference between ponds with confirmed occupancy and those with no confirmed occupancy. We use a logistic regression to examine the effect that individual HSI covariates have on binary pond occupancy results from the eDNA and direct observational survey data. All continuous covariates were standardised prior to analysis. All data analysis was undertaken in R version 4.0.0 (R-Core Team, 2020) with the additional package glmulti (Calcagno & de Mazancourt, 2010) to undertake logistic regression of all model combinations, and Akaike's information criterion (AIC) model selection. We then calculate the relative importance of each of the HSI covariates using the inbuilt Multimodal inference and assessed them as highly supported or somewhat support based on Marchetti et al. (2004).

RESULTS

Naïve occupancy and detectability

Sixty-four of the 489 ponds surveyed were found to be occupied by great crested newts, either through eDNA or direct observational surveys, give a naïve occupancy rate of 0.13.

Occupancy data was generated using both eDNA and direct observational methods at 85 ponds. The results

of the two methodologies concurred 76 % of the time. In five cases ponds were found to be negative using eDNA, but great crested newts were identified using direct observation-based survey methods (naïve error rate of 6 %). Conversely, 15 of the 85 ponds were found to be positive using eDNA but this was not confirmed using observational methods, (naïve error rate of 18 %). Neither of these estimates take into account the potential for false positive eDNA results and they do not take into account instances where both methodologies may have missed the species, suggesting they are both underestimates.

HSI results

Mean HSI score of all ponds surveyed was found to be 0.64 (95 %CI = 0.58-0.69), ranging from 0.19 to 0.98 (N = 489). Overall, 102 ponds (20.86 %) fell into the ‘Poor’ category (HSI < 0.5), 88 (18.00 %) into the ‘Below Average’ category (HSI = 0.5-0.59), 97 (19.84 %) into the ‘Average’ category (HSI = 0.6-0.69), 119 (24.34 %) into the ‘Good’ category (HSI = 0.7-0.79) and 83 (16.97 %) into the ‘Excellent’ category (HSI > 0.8) (Fig. 1).

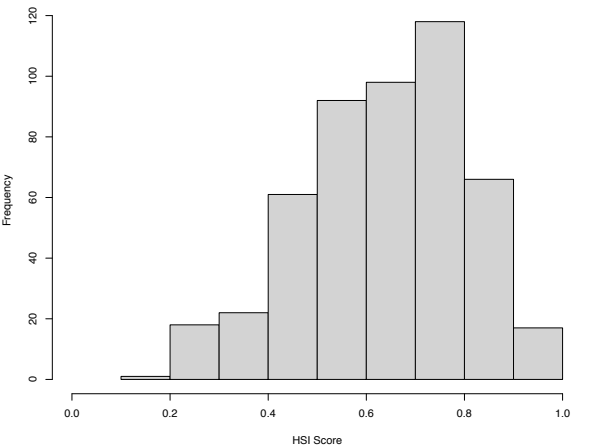
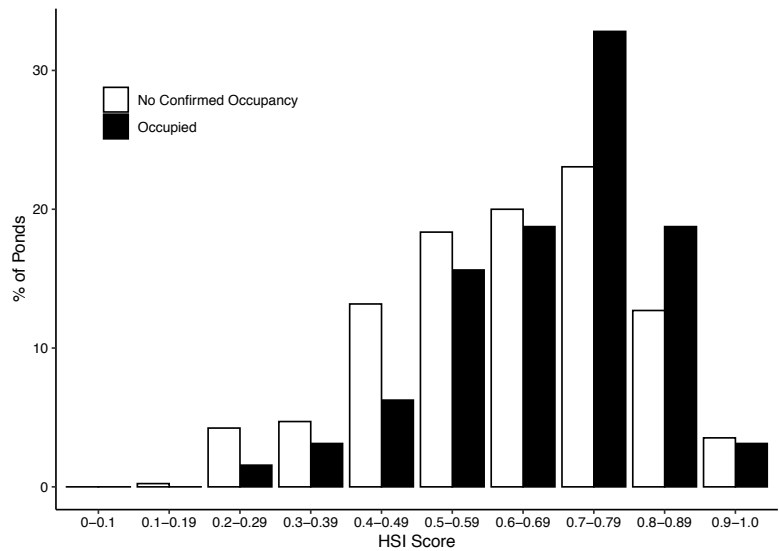


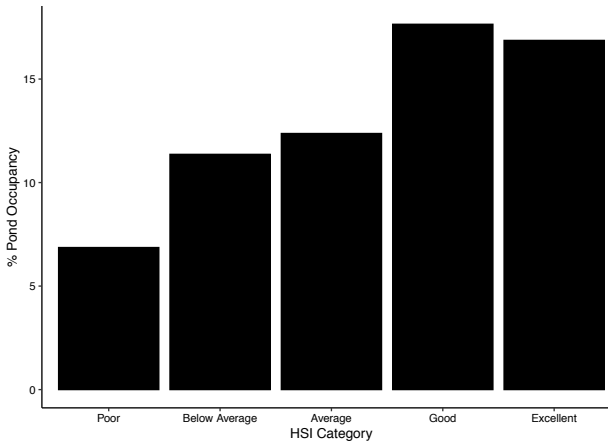
Figure 1. the distribution of HSI score for all ponds surveyed.

HSI scores vs pond occupancy

Mean HSI score in the 64 occupied ponds was found to be 0.68 (standard deviation 0.15), with HSI scores ranging from 0.28 to 0.93. This was comparable to 0.63 for the 425 unoccupied ponds (standard deviation 0.17), with scores ranging from 0.19 to 0.98. Seven of the 64 (10.9 %) occupied ponds had an HSI score below 0.5 suggesting pond quality was ‘Poor’; 10/64 (15.6 %) were of ‘Below Average’ pond quality; 12/64 (18.8 %) were of ‘Average’ pond quality; 21/64 (32.8 %) were of ‘Good’ pond quality; and 14/64 (21.8 %) were of ‘Excellent’ pond quality. This was comparable to ponds with unconfirmed occupancy, where 95 of 425 (22.4 %) had an HSI score below 0.5 indicating ‘Poor’ pond quality; 78/425 (18.4 %) were of ‘Below Average’ pond quality; 85/425 (20.0 %) were of ‘Average’ pond quality; 98/425 (23.1 %) were of ‘Good’ pond quality; and 69/425 (16.2 %) were of ‘Excellent’ pond quality (Fig. 2).



**Figure 2.** Percentage of occupied ponds (black) and ponds with no confirmed occupancy (white) at each 0.1 increments on the HSI Scale.

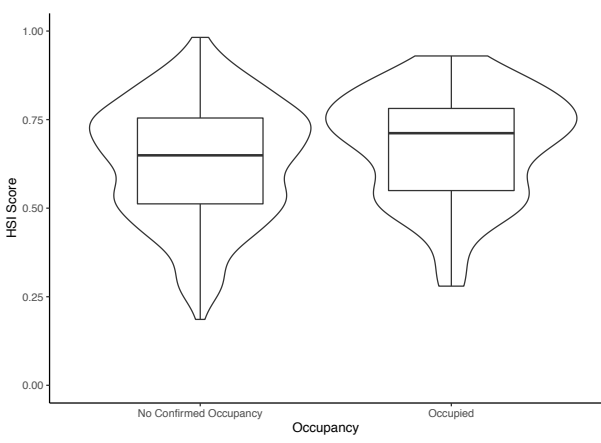


**Figure 3.** Percentage of occupied ponds for great crested newts within each HSI category.

Correspondingly, 7/102 (6.9 %) of ‘Poor’ ponds were occupied, 10/88 (11.4%) ‘Below Average’ ponds occupied, 12/97 (12.4 %) ‘Average’ ponds were occupied, 21/119 (17.6 %) ‘Good’ ponds were occupied, and 14/83 (16.9 %) ‘Excellent’ ponds were occupied (Fig. 3). Consequently, there is a slight increase in pond occupancy in the more favourable HSI categories. A general linear model was used to assess whether pond occupancy by great crested newts was related to HSI score, and a slight positive significant relationship was identified (df = 491, t = 2.06, p = 0.04; Fig. 4).

**Covariate analysis**

We examined the individual effects of the ten HSI predictor variables on predicting pond occupancy. The model with the greatest AIC support was found to only include waterfowl and fish as covariates; however, an additional nine models fell within <2 delta AIC units of the top model (Table S1). Further analysis was undertaken on cumulative AIC importance weights for



**Figure 4.** Great crested newt occupancy compared to the overall HSI score. Occupancy taken from the combined naïve eDNA and direct observational survey results, (n=489).

each of the HSI covariates with waterfowl presence (cumulative AIC weight = 0.8734) strongly supported by the analyses as an important covariate. Fish presence was of secondary importance (cumulative AIC weight = 0.7216), with shade (cumulative AIC weight = 0.4611) having less support. Geographic location, pond area, pond count, macrophyte cover, water quality, pond permanence and terrestrial habitat quality each had a cumulative AIC weighting of <0.4. However, there was very little significance within the model with the greatest AIC support. The only covariate to show any significance was the “Minor” category for waterfowl presence (df = 485; t-value = 2.276; p-value = 0.0233).

**DISCUSSION**

The naïve pond occupancy identified in this data set (0.13) is low when compared to other published occupancy rates for great crested newts in the UK. Sewell et al. (2010) found a naïve occupancy rate for both south-

east England and parts of Wales to be approximately 0.3. However, the results of this study are closer to the naïve national estimates of between 13 % and 18 % from the Freshwater Habitats Trust PondNet study (Ewald, 2018). The naïve occupancy estimates found in this data set suggest that the study area is more in line with the national average than some previous estimates from high-density areas such as south-east England.

These data show that ponds with a higher HSI score are more likely to be occupied, but there is considerable overlap in scores for ponds with and without confirmed occupancy. Ponds with no confirmed occupancy were found to score as high as 0.98, while ponds with a score as low as 0.23 were found to be occupied. The use of pond categories may be useful for presenting results to a non-ecological audience; however, this simplification may be leading to misinterpretation and overreliance on the HSI as an indicator of presence-absence. The use of an HSI score to predict whether a pond will be occupied or not is therefore unwise.

It has been argued - and accepted by some planning authorities - that ponds with low HSI scores can be ruled out for occupancy assessments. Examples of this can be found within planning applications to Aylesbury Vale District (application number 19/00909/APP), Milton Keynes Council (application number 20/01134/FUL), Bedford Borough Council (application number 20/00289/FUL) and Tewksbury District Council (application number P0156/20/FUL) as well as others. However, the HSI was never intended to be used as a surrogate for pond occupancy, but was intended as a risk register of potential great crested newt presence, identifying ponds with appropriate habitat. Here we see that seven of the 102 ponds (7 %) in the ‘Poor’ category (HSI<0.5) were occupied. Although this is lower than the overall occupancy rate of 13 %, we do not consider it to be sufficiently lower to justify ruling out follow-up occupancy assessments. As occupancy at low HSI scores have been observed both by this study and Harper et al. (2019), it would be unwarranted to assume absence from a low HSI score. Nevertheless, we did not observe any occupancy below an HSI of 0.2.

An HSI value of 0.7 or above (‘Good’ or ‘Excellent’ categories) has been arbitrarily used as a cut-off to designate likely breeding habitat (Burgess, 2020; Nature Space Partnership, 2019). This is harder to assess using environmental DNA as the method does not differentiate between life stages. The mean HSI for positive ponds was found to be 0.68 with a standard deviation of 0.151, where 35 of 64 (55 %) ponds fell into the ‘Good’ or ‘Excellent’ categories. In certain circumstances, a cut-off value of 0.7 may therefore be argued as appropriate to define potentially important ponds for great crested newts, but with only 21 % of these ponds occupied, it should still not be used to assume occupancy. Similarly, potential occupancy and breeding should not be ruled out in ponds regarded as being less important as almost half of occupied ponds (N = 29: 45 %) in this study had an HSI score lower than 0.7.

It is evident from these data that both eDNA and observational methodologies for assessing pond occupancy have associated rates of sampling error.

Where both eDNA and observational survey methods were used on the same ponds, the results concurred 76 % of the time. Five instances of ponds were found to be negative in the eDNA survey results but positive using observational methods, and fifteen instances where ponds were negative using observational methods but positive for the eDNA survey. Both observational and eDNA survey methods can suffer from false negative error, but the eDNA technique may be subject to false positive error as well (Ficetola et al., 2015; Griffin et al., 2020; Guillerá-Arroita et al., 2017). A way of quantifying this error is needed to aid in the interpretation of large data sets. A recent publication by Griffin et al. (2020), develops statistical models to identify error rates in eDNA studies, both at the sample collection stage and in the laboratory. The application of modelling to generate error rates will allow confidence levels to be applied to the data. eDNA analysis has a further limitation in that only presence or likely absence data can be generated, whereas some degree of abundance estimate can be generated from observational surveys. This being said, a large-scale assessment of pond occupancy covering hundreds of ponds would be logistically unfeasible using observational methods, whereas eDNA surveys allows for regional or countrywide assessments to be undertaken with relative ease.

The HSI score takes the geometric mean of ten pond level variables which are easily collected within the field (Oldham et al., 2000). The use of the geometric mean leaves all ten equally weighted within the overall HSI score. It is highly unlikely that all ten will be equally important in determining suitability of ponds for great crested newts. Using logistic regression and cumulative importance weights we attempted to look at each of the covariates in terms of their importance to great crested newt occupancy. Within this data set only waterfowl and fish presence were found to be important individually. Waterfowl - particularly at high densities - tend to reduce water quality, increase the turbidity of water and reduce vegetation (Edgar & Bird, 2006; Oldham et al., 2000). In addition to increasing turbidity and removal of submerged vegetation, fish also act as direct predators on great crested newt eggs and larvae (Denoël et al., 2013; Edgar & Bird, 2006; Hartel et al., 2010; McLee & Scaife, 1992; Oldham et al., 2000; Rannap et al., 2009a, 2009b; Skea et al., 2006). It is possible the other covariates are correlated and become important in certain combinations, or in different landscapes other variables dominate (Harper et al., 2020). For example, in species distribution modelling within the south of England, pond density has been found to be the strongest predictor of great crested newt distribution (Bormpoudakis et al., 2016). As such, the importance weights presented here may not be universally applicable, with local conditions influencing covariate importance.

In conclusion both eDNA and observational surveys suffer from sampling error and this needs to be acknowledged in the analysis of any great crested newt occupancy assessment. We have found no evidence to support the use of low HSI scores as a justification to rule out direct occupancy assessment. However, there is some justification for the use of high HSI scores to



identify ponds that are potentially important for great crested newts.

Author Contributions

Hannah Tracey organised and helped to carry out the field surveys and helped tabulate the resulting data. Nick Downs helped to carry out the field surveys and drafted a basic initial manuscript. Andrew Buxton wrote the majority of the final manuscript and carried out the data analysis.

Ethics statement

The data was collected as part of a commercially commissioned great created newt distribution assessment, which was subsequently made available for this analysis. This analysis caused no additional survey or disturbance to ponds or target species. All surveys were undertaken by suitably trained and licenced surveyors following national standard protocols.

Data availability

Raw data is available as a supplementary excel data file “Table S2 Raw Data”. R code is hosted on OSF at the following link: [https://osf.io/uk84t/?view\\_only=31da01f779cc4c408dffad490eba5eb0](https://osf.io/uk84t/?view_only=31da01f779cc4c408dffad490eba5eb0)

ACKNOWLEDGEMENTS

A significant number of people and organisations were involved with this project, to which the authors owe a large debt of gratitude. Although it is not possible to name everyone, of particular note was the project management provided by Nick Henson. The ecologists involved in the survey work presented in this paper were employed by Arcadis, working on behalf of the National Grid. Thanks are due to the National Grid for allowing publication. We would like to thank Richard Griffiths and Jim Foster for constructive comments on an earlier version of the manuscript and NatureSpace Partnership for providing planning application examples. We would like to thank two anonymous reviewers whose comments improved the manuscript.

REFERENCES

Allen, A. W. & Hoffman, R. D. (1984). Habitat suitability index models: Muskrat.

ARG UK. (2010). ARG UK Advice Note 5: Great Crested Newt Habitat Suitability Index.

ARG UK. (2017). ARG UK Advice Note 4 Amphibian disease precautions : a guide for UK fieldworkers.

Baker, J., Beebee, T., Buckley, J., Gent, T., Orchard, D., Bennett, A., Bernhard, T., Brady, L., Coward, D., Driver, B., Foster, J., Gleed-Owen, C., Herder, J. et al. (2011). *Amphibian habitat management handbook*. Amphibian and Reptile Conservation.

Biggs, J., Ewald, N., Valentini, A., Gaboriaud, C., Dejean, T., Griffiths, R., Foster, J., Wilkinson, J., Arnell, A., Brotherton, P., Williams, P. & Dunn, F. (2015). Using eDNA to develop a national citizen science-based monitoring programme for the great crested newt (*Triturus cristatus*). *Biological Conservation* 183, 19–28.

Biggs, J., Ewald, N., Valentini, A., Gaboriaud, C., Griffiths, R., Foster, J., Wilkinson, J., Arnett, A., Williams, P. & Dunn, F. (2014). Analytical and methodological development for improved surveillance of the great crested newt Appendix 5. Technical advice note for field and laboratory sampling of great crested newt (*Triturus cristatus*) environmental DNA.

Bormpoudakis, D., Foster, J., Gent, T., Griffiths, R. A., Russell, L., Starnes, T., Tzanopoulos, J. & Wilkinson, J. (2016). Developing models to estimate the occurrence in the English countryside of great crested newts, a protected species under the Habitats Directive. Defra Project WC1108.

Burgess, K. (2020). Monitoring and Evaluation of the Environmental outcomes of District level Licensing for great crested newts.

Buxton, A. S., Groombridge, J. J. & Griffiths, R. A. (2018). Seasonal variation in environmental DNA detection in sediment and water samples. *PLoS ONE* 13, e0191737.

Buxton, A. S., Groombridge, J. J., Zakaria, N. B. & Griffiths, R. A. (2017). Seasonal variation in environmental DNA in relation to population size and environmental factors. *Scientific Reports* 7, 46294.

Calcagno, V. & de Mazancourt, C. (2010). glmulti: An R package for easy automated model selection with (generalized) linear models. *Journal of Statistical Software* 34(12), 29.

Cresswell, W. J. & Whitworth, R. (2004). An assessment of the efficiency of capture techniques and the value of different habitats for the great crested newt *Triturus cristatus*. In *English Nature* (Issue 576).

Denoël, M., Perez, A., Cornet, Y. & Ficetola, G. F. (2013). Similar Local and Landscape Processes Affect Both a Common and a Rare Newt Species. *PLoS ONE* 85, e62727.

Edgar, P. & Bird, D. R. (2006). Action Plan for the conservation of the crested newt *Triturus cristatus* species complex in Europe - Convention on the Conservation of European Wildlife and Natural Habitats. Standing Committee, 26th meeting, Strasbourg, 27-30 November 2006.

English Nature. (2001). Great crested newt mitigation guidelines. *English Nature*.

Ewald, N. (2018). eDNA monitoring for great crested newts 2018. <https://freshwaterhabitats.org.uk/wp-content/uploads/2019/04/eDNA-Great-Crested-Newt-2018.pdf>

Ficetola, G. F., Pansu, J., Bonin, A., Coissac, E., Giguët-Covex, C., De Barba, M., Gielly, L., Lopes, C. M., Boyer, F., Pompanon, F., Rayé, G. & Taberlet, P. (2015). Replication levels, false presences and the estimation of the presence/absence from eDNA metabarcoding data. *Molecular Ecology Resources* 15, 543–556.

Griffin, J. E., Matechou, E., Buxton, A. S., Bormpoudakis, D. & Griffiths, R. A. (2020). Modelling environmental DNA data; Bayesian variable selection accounting for false positive and false negative errors. *Journal of the Royal Statistical Society. Series C: Applied Statistics* 69, 377–392.

Griffiths, R.A., Raper, S. J. & Brady, L. D. (1996). Evaluation of a standard method for surveying common frogs (*Rana temporaria*) and newts (*Triturus cristatus*, *T. helveticus* and *T. vulgaris*). In *JNCC Report No.* 259.

Griffiths, Richard A. & Inns, H. (1998). Surveying. In T. Gent & S. Gibson (Eds.), *Herpetofauna Workers’ Manual*. Joint Nature Conservation Committee.

Guillera-Arroita, G., Lahoz-Monfort, J. J., van Rooyen, A. R., Weeks, A. R. & Tingley, R. (2017). Dealing with false positive

and false negative errors about species occurrence at multiple levels. *Methods in Ecology and Evolution* 8, 1081–1091.

Harper, L. R., Buxton, A. S., Rees, H. C., Bruce, K., Brys, R., Halfmaerten, D., Read, D. S., Watson, H. V., Sayer, C. D., Jones, E. P., Priestley, V., Mächler, E., Múrria, C. et al. (2018). Prospects and challenges of environmental DNA (eDNA) monitoring in freshwater ponds. *Hydrobiologia* 5.

Harper, L. R., Downie, J. R. & McNeill, D. C. (2019). Assessment of habitat and survey criteria for the great crested newt (*Triturus cristatus*) in Scotland: a case study on a translocated population. *Hydrobiologia* 828, 57–71.

Harper, L. R., Lawson Handley, L., Hahn, C., Boonham, N., Rees, H. C., Lewis, E., Adams, I. P., Brotherton, P., Phillips, S. & Hänfling, B. (2020). Generating and testing ecological hypotheses at the pondscape with environmental DNA metabarcoding: A case study on a threatened amphibian. *Environmental DNA* 2, 184–199.

Hartel, T., Nemes, S., Öllerer, K., Coglniceanu, D., Moga, C. & Arntzen, J. W. (2010). Using connectivity metrics and niche modelling to explore the occurrence of the northern crested newt *Triturus cristatus* (Amphibia, Caudata) in a traditionally managed landscape. *Environmental Conservation* 37, 195–200.

Inns, H. (2009). *Britain’s Reptiles and Amphibians : A Guide to the Reptiles and Amphibians of Great Britain, Ireland and the Channel Islands*. WILDGuides.

Jane, S. F., Wilcox, T. M., Mckelvey, K. S., Young, M. K., Schwartz, M. K., Lowe, W. H., Letcher, B. H. & Whiteley, A. R. (2015). Distance, flow and PCR inhibition: eDNA dynamics in two headwater streams. *Molecular Ecology Resources* 15, 216–227.

Jehle, R. & Arntzen, J. W. (2000). Post-breeding migrations of newts (*Triturus cristatus* and *T. marmoratus*) with contrasting ecological requirements. *Journal of Zoology* 251, 297–306.

Langton, T. E. S., Beckett, C. L. & Foster, J. P. (2001). Great crested newt conservation handbook. In *Froglife*. Froglife.

Marchetti, M. P., Light, T., Moyle, P. B. & Viers, J. H. (2004). Fish invasions in California watersheds : testing hypotheses using landscape patterns. *Ecological Applications* 14, 1507–1525.

McLee, A. G. & Scaife, R. . (1992). The colonisation by great cersted newts (*Triturus cristatus*) of a waterbody following treatment with a piscicide to remove large populaions of sticklebakcs (*Gasterosteus aculeatus*). *British Herpetological Society Bulletin* 42, 6–9.

Natural England. (2015). Guidance: Great crested newts: surveys and mitigation for development projects. <https://www.gov.uk/guidance/great-crested-newts-surveys-and-mitigation-for-development-projects>

Nature Space Partnership. (2019). NatureSpace Partnership. 2019. South Midlands region extension GCN District Licensing project Implementation Strategy.

Oldham, R. S., Keeble, J., Swan, M. J. S. & Jeffcote, M. (2000). Evaluating the suitability of habitat for the great crested newt (*Triturus cristatus*). *Herpetological Journal* 10, 143–155.

Pondnet. (2013). How to collect a water sample to detect great crested newt eDNA Sampling the pond. *Protocol*, 1–4.

R-Core Team. (2020). R: language and environment for statistical computing. R Foundation for Statistical Computing.

Rannap, R., Löhmus, A. & Briggs, L. (2009a). Niche position, but not niche breadth, differs in two coexisting amphibians having contrasting trends in Europe. *Diversity and Distributions* 15, 692–700.

Rannap, R., Löhmus, A. & Briggs, L. (2009b). Restoring ponds for amphibians: A success story. *Hydrobiologia* 634, 87–95.

Rees, H. C., Bishop, K., Middleditch, D. J., Patmore, J. R. M., Maddison, B. C. & Gough, K. C. (2014). The application of eDNA for monitoring of the great crested newt in the UK. *Ecology and Evolution* 4, 4023–4032.

Sewell, D., Beebee, T. J. C. & Griffiths, R. A. (2010). Optimising biodiversity assessments by volunteers: The application of occupancy modelling to large-scale amphibian surveys. *Biological Conservation* 143, 2102–2110.

Sewell, D., Griffiths, R. A., Beebee, T. J. C., Foster, J. & Wilkinson, J. W. (2013). Survey protocols for the British herpetofauna Version 1.0 (Issue March). [http://www.narrs.org.uk/documents/Survey\\_protocols\\_for\\_the\\_British\\_herpetofauna.pdf](http://www.narrs.org.uk/documents/Survey_protocols_for_the_British_herpetofauna.pdf)

Skei, J., Dolmen, D., Rønning, L. & Ringsby, T. (2006). Habitat use during the aquatic phase of the newts *Triturus vulgaris* (L.) and *T. cristatus* (Laurenti) in central Norway: proposition for a conservation and monitoring area. *Amphibia-Reptilia* 27, 309–324.

Thomsen, P. F., Kielgast, J., Iversen, L., Wiuf, C., Rasmussen, M., Gilbert, M. T. P., Orlando, L. & Willerslev, E. (2012). Monitoring endangered freshwater biodiversity using environmental DNA. *Molecular Ecology* 21, 2565–2573.

Thomsen, P. F. & Willerslev, E. (2015). Environmental DNA - An emerging tool in conservation for monitoring past and present biodiversity. *Biological Conservation* 183, 4–18.

U.S. Fish and Wildlife Service. (1976). Habitat Evaluation Procedures ESM 101.

U.S. Fish and Wildlife Service. (1980). Habitat Evaluation Procedures (HEP) ESM 102.

U.S. Fish and Wildlife Service. (1981). Standards for the development of habtiat suitability inindex models for use in teh habitat evaluation procedures (HEP).

Unglaub, B., Steinfartz, S., Drechsler, A. & Schmidt, B. R. (2015). Linking habitat suitability to demography in a pond-breeding amphibian. *Frontiers in Zoology* 12, 9.

Wesche, T. A., Goertler, C. M. & Hubert, W. A. (1987). Modified Habitat Suitability Index Model for Brown Trout in Southeastern Wyoming. *North American Journal of Fisheries Management* 7, 232–237.

Wielstra, B., McCartney-Melstad, E., Arntzen, J. W., Butlin, R. K. & Shaffer, H. B. (2019). Phylogenomics of the adaptive radiation of *Triturus* newts supports gradual ecological niche expansion towards an incrementally aquatic lifestyle. *Molecular Phylogenetics and Evolution* 133, 120–127.

Accepted: 1 February 2021

THE HERPETOLOGICAL JOURNAL  
SUBMISSION PROCESS

The *Herpetological Journal* is an international peer-reviewed publication of the British Herpetological Society, with open-access publication options. *The Journal* has a broad focus relating to behaviour, ecology, evolution, systematics, taxonomy, physiology, anatomy, functional morphology, pathology, natural history, method development and conservation of reptiles and amphibians. All articles should appeal to a general herpetological audience and have a solid grounding in natural history. We are committed to open science and avoiding unconscious biases so moving forward we will operate a double-blind peer review process.

Manuscripts that describe natural history observations, range extensions or checklists are not appropriate submissions (unless they address a bigger question) and would be better suited to our sister publication, *The Herpetological Bulletin*.

The *Herpetological Journal* welcomes contributions in the following categories:

- Full length research articles
- Short communications
- Reviews
- Perspectives/Opinion pieces

Files to be uploaded to the OJS system: Cover Page, Main Text, Tables, Figures, Supplementary Files.

**Cover Page:**  
This will not be seen by peer reviewers in order to comply with a double-blind peer review process. The cover page should include Title, Authors, Author Affiliations, Ethical Statement and Author Contributions (if editors do not deem that authors contributed substantially to the research then the article will be rejected).

**Main Text:**  
Nb. author names or affiliations should not be included in this file.

Full length research articles should be between 2,500 and 6,000 words and include the following sections: Title, Abstract (maximum 300 words), Keywords (five words that are not used in the title), Introduction, Methods, Results, Discussion, Acknowledgements, Data Accessibility, References, Figure captions, Table captions. The word limit excludes Data Accessibility and References. There are no limits to the number of figures and tables.

Short communications should be less than 2,500 words and include the following sections: Title, Abstract (maximum 250 words), Keywords (five words not used in the title), Main Text (NOT separated into Introduction, Methods, Results and Discussion), Acknowledgements, Data Accessibility, References, Figure captions, Table captions. The word limit excludes Data Accessibility, References, and Figure and Table captions. Short communications can have a maximum of ONE figure and ONE table.

Reviews are either solicited by editors or a short email enquiry should be sent to the Editor-in-Chief (bhsherpetologicaljournal@gmail.com) to enquire about the suitability of a proposed review. Reviews should be between 2,500 and 6,000 words. Section headings can be specified at the authors discretion.

Perspectives/Opinion pieces will be considered if they address a new or controversial topic/idea, or if they are comments about newly published articles in *The Herpetological Journal*. Perspectives/Opinion pieces should be a maximum of 1,500 words (excluding references) and can include ONE figure and ONE table.

**Tables:**  
Tables should be provided in a separate Word file. Tables should be numbered in Arabic numerals, e.g. Table 1. Tables should be as simple as possible and typed double-spaced on separate sheets with a title/short explanatory paragraph above the table. Horizontal and vertical lines should be avoided, as should tables that split over more than one page or that need to be set in landscape format.

**Figures:**  
Figures should initially be submitted in a single Word or PDF file. Graphs, line drawings and photographs should be numbered in sequence in Arabic numerals, e.g. Figure 1. If a figure has more than one part, each should be identified as (a), (b), etc. Figure captions should be included at the end of the main text. After acceptance figures should be submitted as separate image or pdf files with a minimum resolution of 300dpi and a maximum file size of 5MB.

**Supplementary Data/Files:**  
To conform with an open science process, it will be necessary for datasets, code, supplementary figures etc. to be deposited in an online repository (e.g. <https://osf.io/>) and made available after publication. At the initial submission stage, at a minimum, any code and supplementary figures should be uploaded in the submission portal for review. Any new taxonomic changes should be recorded on ZooBank.

It is a fundamental condition that submitted manuscripts have not been published and will not be simultaneously submitted or published in another journal. However, as a journal we do support the submission of articles on preprint servers (e.g., bioRxiv) as long as the preprints are linked to final published articles.

By submitting a manuscript, the authors agree that the copyright for their article is transferred to the publisher if and when the article is accepted for publication. The copyright covers the exclusive rights to reproduce and distribute the article, including reprints and photographic reproductions. Permission to use images after publication will almost always be granted but must be sought in advance from the Editors.

Papers should be written in British English (including figure labels) and spelling should be that of the Oxford English Dictionary.

Times and dates should conform to the following formats: for time of day use 0900, 1000 etc; for dates

use 7 July 2017 etc. Please avoid using bold text, all caps or small caps for emphasis. If emphasis is required, use italics. Common names should be in lower case unless a proper noun is used.

All submissions must adhere to the British Herpetological Society's Ethical Policy and Guidelines, which can be found here – <https://www.thebhs.org/images/stories/BHS-ethicspolicy.pdf>.

**Open Access Policy:** *The Herpetological Journal* supports "green" open access, as outlined by the Research Councils UK, to facilitate deposition of articles e.g. at institutional repositories. *The Herpetological Journal* also offers the option of "gold" open access for individual articles (free of charge for members of the British Herpetological Society, and at an article processing charge of £97 for non-members).

**REFERENCE STYLE**  
**CITATION IN TEXT:**

- Chronological then alphabetical
- Use “et al.” (not italicised) for more than two authors
- Last name (s) and year separated by comma
- Names separate by “&”
- References separated by semicolon

Ex. 1: (Heyer et al., 1988; Weygoldt et al., 1989; Eterovick et al., 2005)  
Ex. 2: (Smith et al., 2004; Jones & Smith, 2008)  
Ex. 3: (Smith et al., 2015)  
Ex. 4: “Although Smith et al. (2008) did not include -”  
Ex. 5: "- as observed by Smith & Jones (2017)”

**REFERENCES**

- Authors
- Last name separated from initials by comma
- Initials capitalised and separated by period (no space)
- Names separated by commas
- Last name separated by “&”

Ex.: Smith, A.H., Jones, R.D. & Lloyd, K.A.  
Ex.: Smith, A.H. & Jones, R.D.

Year:

- In parentheses, followed by a full stop.

Title:

- Only first letter capitalised except book titles (in this case, All First Letters Capitalised).

Journal:

- Journal name should be written in full, italicised, followed by a comma
- Volume and pages separated by comma and ending with full stop (not italicised).

Journal article:

- Authors. (Year). Title. *Journal*, Volume (Issue), xx–xx.

Book:

- Authors. (Year). Book Title. City: Country. Xxx p.

Book chapter:

- Authors. (Year). Chapter title. In: *Book Title*, Book editor (s). (Ed./Eds.) City: Country. Xxx p.

Ex. 1:  
Lebboroni, M. & Corti, C. (2006). Road-killing of lizards and traffic density in central Italy. In: *Herpetologia Bonnensis II: Proceedings of the 13th Ordinary General Meeting of Societas Europaea Herpetologica*, 81–82. Vences, M., Köhler, J., Ziegler, T. & Böhme, W. (eds). Bonn: Societas Europaea Herpetologica.

Ex. 2:  
Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989). Preparation and Analysis of Eukaryotic Genomic DNA. In: *Molecular Cloning: A Laboratory Manual*, 2nd Eds. Cold spring Harbor Laboratory Press, New York, USA.

Websites:

Lang, J., Chowfin, S. & Ross, J.P. (2019). *Gavialis gangeticus*. The IUCN Red List of Threatened Species 2019: e.T8966A149227430. Downloaded on 3 October 2019. <http://dx.doi.org/10.2305/IUCN.UK.2019-1.RLTS.T8966A149227430.en>.

**All contributions should be addressed to the Scientific Editor:**

Simon T. Maddock, University of Wolverhampton, UK.  
*E-mail:* [bhsherpetologicaljournal@gmail.com](mailto:bhsherpetologicaljournal@gmail.com)

**Associate Scientific Editors:**

Annemarieke Spitzen, Anthony Herrell, Anyelet Valencia-Aguilar, Ben Tapley, Deepak Veerappan, Diogo Borges Provete, Gabriella Bittencourt, Inga Zeisset, Jim Labisko, John Vanek, Jose Valdez, Lewis Campbell, Luis San José, Mirco Solé, Rachael Antwis, Richard Brown, Robert Jehle, Sam Cruickshank, Simon Loader.

**Managing Editor:**

Sarah Berry ([info@sarahberryonline.com](mailto:info@sarahberryonline.com))

**Advertisements:**

*The Herpetological Journal* accepts advertisements subject to approval of contents by the Managing Editor, to whom enquiries should be addressed.

*Herpetological Journal* website: <https://www.thebhs.org/publications/the-herpetological-journal>



